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<b>(21) International Application Number:</b> PCT/GB96/02980 <b>(22) International Filing Date:</b> 3 December 1996 (03.12.96) <b>(71) Applicant (for all designated States except US):</b> MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FERSHT, Alan, Roy [GB/GB]; MRC Unit for Protein Function and Design, University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB). ZAHN, Ralph [GB/GB]; MRC Unit for Protein Function and Design, University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB). ALTAMIRANO, Myriam, Marlene [MX/GB]; 302 Hills Road, Cambridge CB2 2QG (GB). <b>(74) Agent:</b> MASCHIO, Antonio; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> CHAPERONE FRAGMENTS  <b>(57) Abstract</b>  An <i>E. coli</i> GroEL polypeptide fragment (191–376) being the optical domain is coupled to a chromatographic resin (Ni–NTA Agarose). A denatured solution of cyclophilin A of known specific activity prior to denaturation is loaded onto a column of the GroEL polypeptide resin and eluted in a refolding buffer. The cyclophilin A is refolded (reconditioned) such that its specific activity is > 100 % of what it was prior to denaturation.		

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Chaperone Fragments

The present invention relates to chaperone polypeptides  
5 which are active in the folding and maintenance of  
structural integrity of other proteins. The invention  
also relates to nucleic acids encoding chaperone  
polypeptides, vectors comprising these nucleic acids,  
host cells modified with the nucleic acids or vectors so  
10 as to express the chaperone polypeptides. The invention  
further relates to methods of making chaperone  
polypeptides whether by synthetic or recombinant means,  
pharmaceutical compositions comprising the chaperone  
polypeptides or nucleic acids encoding the same and the  
15 use of chaperone polypeptides in the treatment of disease  
or in the reconditioning of biologically active  
materials. The invention also relates to antibodies  
reactive against chaperone polypeptides and their use in  
medicine and diagnostics.

20

Chaperones are in general known to be large multisubunit  
protein assemblies essential in mediating polypeptide  
chain folding in a variety of cellular compartments.  
Families of chaperones have been identified, for example  
25 the chaperonin hsp60 family otherwise known as the cpn60  
class of proteins are expressed constitutively and there  
are examples to be found in the bacterial cytoplasm  
(GroEL), in endosymbiotically derived mitochondria  
(hsp60) and in chloroplasts (Rubisco binding protein).  
30 Another chaperone family is designated TF55/TCP1 and  
found in the thermophilic archaea and the evolutionarily  
connected eukaryotic cytosol. A comparison of amino acid  
sequence data has shown that there is at least 50%  
sequence identity between chaperones found in

prokaryotes, mitochondria and chloroplasts (Ellis R J and Van der Vies S M (1991) *Ann Rev Biochem* 60: 321-347).

A typical chaperonin is GroEL which is a member of the  
5 hsp60 family of heat shock proteins. GroEL is a  
tetradecamer wherein each monomeric subunit (cpn60m) has  
a molecular weight of approximately 57kD. The  
tetradecamer facilitates the *in vitro* folding of a number  
of proteins which would otherwise misfold or aggregate  
10 and precipitate. The structure of GroEL from *E. coli* has  
been established through X-ray crystallographic studies  
as reported by Braig K et al (1994) *Nature* 371: 578-586.  
The holo protein is cylindrical, consisting of two seven-  
membered rings that form a large central cavity which  
15 according to Ellis R J and Hartl F U (1996) *FASEB Journal*  
10: 20-26 is generally considered to be essential for  
activity. Some small proteins have been demonstrated to  
fold from their denatured states when bound to GroEL  
(Gray T E and Fersht A R (1993) *J Mol Biol* 232: 1197-  
20 1207; Hunt J F et al (1996) *Nature* 379: 37-45; Weissman J  
S et al (1996) *Cell* 84: 481-490; Mayhew M et al (1996)  
*Nature* 379: 420-426; Corrales F J and Fersht A R (1995)  
*Proc Nat Acad Sci* 92: 5326-5330) and it has been argued  
that a cage-like structure is necessary to sequester  
25 partly folded or assembled proteins (Ellis R J and  
Hartl F U (1996) *supra*.

The entire amino acid sequence of *E. coli* GroEL is also  
known (see Braig K et al (1994) *supra*) and three domains  
30 have been ascribed to each cpn60m of the holo chaperonin  
(tetradecamer). These are the intermediate (amino acid  
residues 1-5, 134-190, 377-408 and 524-548), equatorial  
(residues 6-133 and 409-523) and apical (residues 191-  
376) domains.

Monomers of GroEL have been induced by urea or pressure, but they are inactive and have to reassociate to form the central cavity in order to facilitate the refolding of  
5 rhodanese (Mendoza J A et al (1994) J Biol Chem 269: 2447-2451; Ybarra J and Horowitz P M (1995) J Biol Chem 270: 22962-22967).

GroEL facilitates the folding of a number of proteins by  
10 two mechanisms; (1) it prevents aggregation by binding to partly folded proteins (Goloubinoff P et al (1989) Nature 342: 884-889; Zahn R and Plückthun A (1992) Biochemistry 31: 3249-3255), which then refold on GroEL to a native-like state (Zahn R and Plückthun A (1992) Biochemistry  
15 31: 3249-3255; Gray T E and Fersht A R (1993) J Mol Biol 232: 1197-1207); and (2) it continuously anneals misfolded proteins by unfolding them to a state from which refolding can start again (Zahn R et al (1996) Science 271: 642-645). Some mutations in the apical  
20 domain led to a decrease in polypeptide binding (Fenton W A et al (1994) Nature 371: 614-619), suggesting that this domain is involved in the binding of polypeptides. Electron microscopy suggests that denatured protein binds to the inner side of the apical end of the GroEL-cylinder  
25 (Chen S et al (1994) Nature 371: 261-264). The equatorial domain has been shown from the 2.4 Å crystal structure of ATPγS-ligated GroEL (Boisvert D C et al (1996) Nature Structure Biology 3: 170-177) and  
mutagenesis studies (Fenton W A et al (1994) Nature 371:  
30 614-619) to have the nucleotide binding sites. Binding and hydrolysis of ATP is cooperative (Bochkareva E S et al (1992) J Biol Chem 267: 6796-6800; Gray T E and Fersht A R (1991) FEBS Lett 292: 254-258), and lowers the

affinity for polypeptides (Jackson G S et al (1993) Biochemistry 32: 2554-2563). Most of the intermolecular contacts between the subunits of GroEL are between the equatorial domain. The intermediate domain connects the  
5 other two domains, transmitting allosteric effects (Braig K et al (1994) Nature 371: 578-586; Braig K et al (1995) Nature Struct Biol 2: 1083-1094).

The crystal structure of GroEL shows unusually high *B*-  
10 factors for the apical domain compared with the equatorial or intermediate domain, and the *B*-factors vary considerably within the domain (Braig K et al (1994) Nature 371: 578-586; Braig K et al (1995) Nature Struct Biol 2: 1083-1094; Boisvert D C et al (1996) Nature  
15 Structure Biology 3: 170-177). The high overall *B*-factor seems to result from a static disorder within the asymmetric unit and probably throughout the crystals of GroEL, and has been attributed to rigid-body movements generated by hinge-like  $\beta$ -sheets in the intermediate  
20 domain. Regions of high flexibility have also been observed in the 2.8Å structure of the co-chaperonin GroES (Hunt J F et al (1996) Nature 379: 37-45). A mobile loop has been shown to be directly involved in ADP-dependent binding to the apical domain (Landry S J et al (1993)  
25 Nature 364: 255-258). Binding of GroES leads to a conformational change of GroEL and a concomitant enlargement of the GroEL-cavity (Chen S et al (1994) Nature 371: 261-264), in which the encapsulated polypeptide substrate can refold to a native-like state  
30 without the danger of aggregation (Martin J et al (1993) Nature 366: 228-233; Weissman J S et al (1995) Cell 83: 577-587).

Monomeric forms of GroEL have been induced by site-directed mutagenesis and expressed and although these bind to rhodanese they do not affect its refolding (White Z W et al (1995) J Biol Chem 270: 20404-20409).

5

Yoshida et al (1993) FEBS 336: 363-367 report that a 34kD proteolytic fragment of *E. coli* GroEL which lacks 149 NH<sub>2</sub>-terminal residues and ~93 COOH-terminal residues (GroEL 150-456) facilitates refolding of denatured  
10 rhodanese in the absence of GroES and ATP. Although the proteolytic fragment GroEL 150-456 elutes as a monomer during gel filtration, it still comprises the apical domain and significant portions of the intermediate and equatorial domains, the latter of which determine the  
15 intersubunit contacts of GroEL (Braig K et al (1994) *supra*), thus allowing transient formation of the central cavity thereby accounting for the chaperonin activity which is observed.

20 In any event, the mode of rhodanese refolding by GroEL 150-456 is very different from that brought about by the holo protein; the yield of productive refolding is low, folding is rapidly saturated with time, and it is not affected by GroES and ATP. Efficient release and folding  
25 requires the hydrolysis of ATP (Landry S J et al (1992) Nature 355: 455-457; Gray T E and Fersht A R (1992) FEBS Lett 282: 254-258; Jackson G S et al (1993) Biochemistry 32: 2554-2563; Todd M et al (1993) Biochemistry 32: 8560-8567.)

30

EP-A-0 650 975 (NIPPON OIL CO LTD) discloses chaperoin molecules and a method of refolding denatured proteins using GroEL chaperonin 60 monomers (cpn60m) obtained from *Thermus thermophilus*. The holo-chaperonin was first

extracted and then purified from the bacterial source according to the method of Taguchi et al (1991) J Biol Chem 266: 22411-22418. The cpn60m was then produced by treatment of the holo-chaperonin with trifluoroacetic acid (TFA) followed by reverse phase (rp) HPLC of the resulting denatured protein. A peak fraction containing the approximately 57kD cpn60m was obtained. The refolding activity of the cpn60m was assayed in solution by monitoring the regain in activity of inactivated rhodanese, which in specific activity terms amounted to about only 25% of the specific activity of the rhodanese prior to inactivation. When background spontaneous rhodanese refolding is subtracted then there is only an approximately 20% refolding activity.

As well as cpn60m, EP-A-0 650 975 also discloses the use of an approximately 50kD N-terminal deletion fragment of cpn60m wherein the N-terminal amino acid residues up to (but not including) the Thr residue at position 79 are removed by proteolysis. This 50kD fragment showed an approximately 35% (about 30% when background is subtracted) rhodanese refolding activity when in solution.

Taguchi H et al (1994) J Biol Chem 269: 8529-8534 is a scientific report on which the invention of EP-A-0 650 975 is based. A transiently formed GroEL tetradecamer (the holo-chaperonin) was perceived to exist when the chaperonin monomers are present in solution. Consequently, the refolding activity of these preparations can be seen to be caused by the presence of holo chaperonin, not monomers. To test this, Taguchi et al immobilised cpn60m to a chromatographic resin to exclude the possibility of holo chaperonin formation.



When immobilised and therefore when in truly monomeric form, cpn60m exhibited only about 10% rhodanese refolding activity.

- 5 The refolding of rhodanese has been a common and convenient assay to determine chaperonin activity but it has been observed that there are significant problems with the assay which cast serious doubt on existing assertions of refolding activity based on this assay.
- 10 The fact is that rhodanese refolds spontaneously in the absence of molecular chaperones with the yield of refolded rhodanese increasing progressively as the rhodanese concentration decreases (see Taguchi *et al* (1994) *supra*). The 10% of rhodanese refolding activity
- 15 reported in EP-A-0 650 975 for immobilised (truly monomeric) cpn60m is therefore too close to the spontaneous regain of activity by rhodanese to demonstrate that any monomeric chaperonin has a refolding activity towards proteins generally, let alone cpn60m and
- 20 rhodanese.

- Alconada A and Cuezva J M (1993) TIBS 18: 81-82 suggested that an "internal fragment" of GroEL may possess a chaperone activity on the basis of amino acid sequence
- 25 similarity between the altered mRNA stability (ams) gene product (Ams) of *E. coli* and the central part of GroEL. The ams locus is a temperature-sensitive mutation that maps at 23 min on the *E. coli* chromosome and results in mRNA with an increased half-life. The ams gene has been
- 30 cloned, expressed and shown to complement the ams mutation. The gene product is a 149-amino acid protein (Ams) with an apparent molecular weight of 17kD.

Chanda P K et al (1985) J Bacteriol 161: 446-449 found that a 17kD protein fragment corresponding to part of the L gene of the groE operon, when expressed in *E. coli* ams mutants restores the wild-type phenotype. This 17kD  
5 fragment was suggested as being an isolated, functional chaperonin protein module. The amino acid sequences of three chaperonins (*E. coli* GroEL, ribulose biphosphate carboxylase (RUBPC) subunit-binding protein from *Triticum aestivum* and *Saccharomyces cerevisiae*, mitochondrial  
10 hsp60) were compared with the sequence of Ams. Residues 307-423 were found to correspond substantially between Ams and GroEL. These residues comprise nearly equivalent portions of both the intermediate and apical domains of GroEL.  
15  
The sequence alignments of Ams protein with the chaperonins noted above reveals a striking similarity (98%) between the amino-terminal four-fifths of Ams and the central part (approximately one-fifth) of *E. coli*  
20 GroEL chaperonin. The 50% sequence similarity between the Ams amino terminal region and the two other chaperonins is in line with the reported identity among the chaperonin family. The carboxy-terminal part of the Ams protein showed no similarity with chaperonins (<10%  
25 homology).

The present inventors have identified a need for a simple, truly monomeric chaperone molecule which is of defined sequence and structure and which can efficiently  
30 and reproducibly refold, renature, reactivate or recondition proteins from a range of sources in the absence of added cofactor or other agents and without the need to associate to form a holo chaperonin. A problem which the invention seeks to solve is therefore how to

provide an active portion or fragment of a chaperone in truly monomeric form so as to promote a useful and efficient reagent for the refolding, renaturing or reconditioning of biological molecules, particularly proteins. A further object is the provision of such a monomeric form at minimum size.

In first aspect the present invention provides a chaperone polypeptide having an amino acid sequence selected from at least amino acid residues 230-271 but no more than residues 150-455 or 151-456 of a GroEL sequence substantially as shown in Figure 7, or a corresponding sequence of a substantially homologous chaperone polypeptide, or a modified, mutated or variant thereof having chaperone activity.

The amino acid sequence is preferably selected from at least amino acid residues 193-337, more preferably 191-345, even more preferably 191-376 but no more than residues 151-455. The invention therefore includes polypeptides being GroEL amino acid residues 230-271, 230-272 ...et seq... 230-455 and in like manner residues 230-271, 229-271 ...et seq... 151-271. Also, residues 230-271, 229-272 ...et seq... 151-351, 151-352 ...et seq... 151-455. All amino acid sequences of 42 or more residues comprising at least contiguous residues 230-271 and not exceeding 151-455 are within the scope of this aspect of the invention eg 171-423 or 166-406.

There are four key properties that may characterise a protein as a molecular chaperone (1) suppression of aggregation during protein folding; (2) suppression of aggregation during protein unfolding; (3) influence on

the yield and kinetics of folding; and (4) effects exerted at near stoichiometric levels.

Chaperone activity may be determined in practice by an  
5 ability to refold cyclophilin A but other suitable  
proteins such as glucosamine-6-phosphate deaminase or a  
mutant form of indoleglycerol phosphate synthase (IGPS)  
(amino acid residues 49-252) may be used. A rhodanese  
refolding assay may also be used. Details of suitable  
10 refolding assays are described in more detail in the  
specific examples provided hereinafter.

In second aspect the invention provides monomeric  
polypeptide having chaperone activity and incapable of  
15 multimerisation in solution.

In third aspect the invention provides a chaperone  
polypeptide which, when in solution, remains monomeric  
and has the ability to refold, reactivate or recondition  
20 proteins, said polypeptide including the protein binding  
active site motif:

1 X X X 2 X X 3 4 X X 5 X X X X X X X X X X X X X X X  
6 X X 7 8 X 9 10 11 X X 12 X 13 14

25

wherein 1 is selected from amino acid residues:  
I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

30 wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;  
 wherein 10 is selected from: V, L, I, F or H;  
 wherein 11 is selected from: N, S or L;  
 wherein 12 is selected from: R, K, N, Q, L or S;  
 5 wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
 wherein 14 is selected from: V, I, L, F, D or T; and

wherein the X's represent a peptide bond or bonds or  
 at least one amino acid residue,

10

or a functional variant thereof in which one or more of  
 the numbered amino acid residues 1 to 14 has undergone a  
 conservative substitution.

15 In fourth aspect the invention provides a chaperone  
 polypeptide which, when in solution, remains monomeric  
 and has the ability to refold, reactivate or recondition  
 proteins, said polypeptide including at least one protein  
 binding active site motif moiety selected from:

20

(a) 1 X X X 2 X X 3 4 X and

(b) X 5 X X X X X X X X X X X X X X 6 X X 7 8 X  
 9 10 11 X X 12 X 13 14

25

wherein 1 is selected from amino acid residues:

I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

30 wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

- wherein 9 is selected from: V, L, I or A;  
wherein 10 is selected from: V, L, I, F or H;  
wherein 11 is selected from: N, S or L;  
wherein 12 is selected from: R, K, N, Q, L or S;  
5 wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
wherein 14 is selected from: V, I, L, F, D or T; and

wherein X is at least one amino acid residue,

wherein 9 is selected from: V, L, I or A;  
 wherein 10 is selected from: V, L, I, F or H;  
 wherein 11 is selected from: N, S or L;  
 wherein 12 is selected from: R, K, N, Q, L or S;  
 5 wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
 wherein 14 is selected from: V, I, L, F, D or T; and

wherein the X's represent a peptide bond or bonds or  
 at least one amino acid residue,

10

or a functional variant thereof in which one or more of  
 the numbered amino acid residues 1 to 14 has undergone a  
 conservative substitution.

15 In fourth aspect the invention provides a chaperone  
 polypeptide which, when in solution, remains monomeric  
 and has the ability to refold, reactivate or recondition  
 proteins, said polypeptide including at least one protein  
 binding active site motif moiety selected from:

20

(a) 1 X X X 2 X X 3 4 X and

(b) X 5 X X X X X X X X X X X X X X 6 X X 7 8 X  
 9 10 11 X X 12 X 13 14

25

wherein 1 is selected from amino acid residues:  
 I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

30 wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;  
wherein 10 is selected from: V, L, I, F or H;  
wherein 11 is selected from: N, S or L;  
wherein 12 is selected from: R, K, N, Q, L or S;  
5 wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
wherein 14 is selected from: V, I, L, F, D or T; and

wherein X is at least one amino acid residue,  
10 or a functional variant thereof in which one or more of  
the numbered amino acid residues 1 to 14 has undergone a  
conservative substitution.

In fifth aspect the claim provides a chaperone  
15 polypeptide which, when in solution, remains monomeric  
and has the ability to refold, reactivate or recondition  
proteins, said polypeptide including the protein binding  
active site motif:

20 I X X X L X X L E X X A X X X X X X X X X X X X X X X  
E X X A T X V V N X X R X I V

wherein X is at least one amino acid residue, or a  
functional variant thereof in which one or more of the  
25 specified amino acid residues has undergone a  
conservative substitution.

In sixth aspect the claims provides a chaperone  
polypeptide which, when in solution, remains monomeric  
30 and has the ability to refold, reactivate or recondition  
proteins, said polypeptide including at least one protein  
binding active site motif moiety selected from:

(a) I X X X L X X L E X



(b) X A X X X X X X X X X X X X X E X X A T X  
V V N X X R X I V

- 5 wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.
- 10 A conservative substitution is the replacement of one amino acid residue for another chemically or functionally similar amino acid residue such that the function of the polypeptide overall remains substantially unchanged.
- 15 The terms "refold", "reactivate" and "recondition" are not intended as being mutually exclusive. For example, an inactive protein, perhaps denatured using urea may have an unfolded structure. This inactive protein may then be refolded with a polypeptide of the invention
- 20 thereby reactivating it. In some circumstances there may be an increase in the specific activity of the refolded/reactivated protein compared to the protein prior to inactivation/denaturation: this is termed "reconditional".
- 25 Preferably, the active site motif or an active site motif moiety includes the conserved sequence:

PLL(V)I(V)IA(S)EDV(I)EGEAL

30

in which amino acid symbols in parenthesis are alternatives to the immediately preceding symbol reading left to right.

In seventh aspect the invention provides monomeric polypeptide having chaperone activity and incapable of multimerisation characterised in that in the absence of ATP the polypeptide has a protein refolding activity of more than 50%, preferably 60%, even more preferably 75%, said refolding activity being determined by contacting the polypeptide with an inactivated protein of known specific activity prior to inactivation, and then determining the specific activity of the said protein after contact with the polypeptide, the % refolding activity being:

$$\frac{\text{specific activity of protein after contact with polypeptide}}{\text{specific activity of protein prior to inactivation}} \times \frac{100}{1}$$

Preferably, the chaperone activity is determined by the refolding of cyclophilin A. More preferably, 8M urea denatured cyclophilin A (100 $\mu$ M) is diluted into 100mM potassium phosphate buffer pH7.0, 10mM DTT to a final concentration of 1 $\mu$ M and then contacted with at least 1 $\mu$ M of said polypeptide at 25°C for at least 5 min, the resultant cyclophilin A activity being assayed by the method of Fischer G et al (1984) Biomed Biochim Acta 43: 1101-1111.

The polypeptide is preferably an hsp60 polypeptide, preferably a GroEL polypeptide.

In eighth aspect the invention provides a polypeptide as claimed in any preceding claim which comprises at least an amino acid sequence selected from GroEL residues:

- (a) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336, 191-337, 191-338, 191-339,

- 5 191-340, 191-341, 191-342, 191-343, 191-344, 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352, 191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360, 191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368, 191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-376, or
- 10 (b) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336, 192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344, 192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352, 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360, 192-361, 15 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368, 192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-376, or
- 20 (c) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336, 193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344, 193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352, 193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360, 193-361, 25 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368, 193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-376, or
- 30 (d) 230-271, 229-271, 229-272, 228-272, 228-273, ...et seq... 194-328, 194-329, or

the equivalent residues of substantially homologous chaperonins, or a modified, mutated or variant sequence thereof.

A preferred polypeptide has the amino acid sequence 191-345 or 191-376, more preferably 193-337 of GroEL, or the equivalent residues of substantially homologous  
5 chaperonins, or a modified, mutated or variant sequence thereof.

The polypeptide preferably has a molecular weight of less than 34kDa.

10

"Modifications" include chemically modified polypeptides for example. "Variants" include, for example, naturally occurring variants of the kind to be found amongst a population of hsp60 chaperonin harbouring organisms/cells  
15 as well as naturally occurring polymorphisms or mutations. "Mutations" may also be introduced artificially by processes of mutagenesis well known to a person skilled in the art.

20 In being "substantially homologous" peptides may have at least 50% amino acid sequence homology with the specified GroEL amino acid sequences, preferably at least 60% homology and more preferably 75% homology. Homology may of course also reside in the nucleotide sequences for the  
25 polypeptide which may be at least 50%, preferably at least 60% homologous and more preferably 75% homologous with the nucleotide sequence encoding the specified GroEL amino acid residues.

30 The hsp60 class of chaperonin proteins are generally homologous in structure and so there are therefore conserved or substantially homologous amino acid sequences between the members of the class. GroEL is just an example of an hsp60 chaperonin protein; other

suitable proteins having an homologous apical domain may be followed.

The list was compiled from the OWL database release 28.1.

- 5 The sequences listed below show clear homology to apical domain (residues 191-375) in PDB structure pdb1grl.ent.

OWL is a non redundant database merging SWISS-PROT, PIR (1-3), GenBank (translation) and NRL-3D.

190-374 CH60\_ECOLI 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(AMS). - ESCHERICHIA 190-374 CH60\_SALTI 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - SALMONELLA TYPHI. 191-375 S56371 GroEL protein - Escherichia coli 190-374 CH60\_LEPIN 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(HEAT SHOCK 58 KD PRO 191-375 S47530 groEL protein - Porphyromonas gingivalis 190-374 LPNHTPBG LPNHTPBG NID:g149691 - Legionella pneumophila (strain SVir)(library: 189-373 CH60\_ACTAC 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - ACTINOBACILLUS ACT 191-375 JC4519 heat-shock protein GroEL - Pasteurella multocida 191-375 CH60\_BRUAB 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - BRUCELLA ABORTUS. 191-375 CH60\_HAEIN 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - HAEMOPHILUS INFLUE 190-373 CH60\_CAUCR 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - CAULOBACTER CRESCE 190-374 CH60\_AMOPS 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - AMOEBA PROTEUS SYM 191-375 CH60\_HAEDU 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - HAEMOPHILUS DUCREY 191-375 CH61\_RHIME 60 KD CHAPERONIN A (PROTEIN CPN60 A)(GROEL PROTEIN A). - RHIZOBIUM ME 190-374 CH60\_LEGMI 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(58 KD COMMON ANTIGEN 191-375 CH60\_YEREN 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(HEAT SHOCK PROTEIN 6) 190-374 CH 63\_BRAJA 60 KD CHAPERONIN 3 (PROTEIN CPN60 3)(GROEL PROTEIN 3). - BRADYRHIZOBI 191-375 CH60\_PORGI 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - PORPHYROMONAS GING 191-375 S52901 heat shock protein 60K - Yersinia enterocolitica 191-375 S26423 heat shock protein 60 - Yersinia enterocolitica 191-375 RSU373691 RSU37369 NID: g1208541 - Rhodobacter sphaeroides strain=HR. 190-374 CH62\_BRAJA 60 KD CHAPERONIN 2 (PROTEIN CPN60 2)(GROEL PROTEIN 2). - BRADYRHIZOBI 191-375 CH60\_ACYPS 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(SYMBIONIN). - ACYRTH 191-375 CH63\_RHIME 60 KD CHAPERONIN C (PROTEIN CPN60 C)(GROEL PROTEIN C). - RHIZOBIUM ME 191-375 YEPHSPCRP1 YEPHSPCRP NID: g466575 - Yersinia enterocolitica DNA. 191-375 CH60\_BORPE 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - BORDETELLA PERTUSS 189-373 BRUGRO1 BRUGRO NID: g144106 - Brucella aabortus (library: lambda-2001) DNA. 191-375 CH60\_PSEAE 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - PSEUDOMONAS AERUGI 190-374 CH60\_BARBA 60 KD CHAPERONIN (PROTEIN CPN60)(IMMUNOREACTIVE PROTEIN BB65)(IMMUNO 191-375 BAOBB63A BAOBB63A NID: g143845 - Bartonella bacilliformis (library: ATCC 35685) 189-373 CH60\_BACST 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - BACILLUS STEAROTHE 188-372 190-373 CH60\_BORBU 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - BORRELIA BURGDORFE 224-408 S26583 chaperonin hsp60 - maize 190-373 A49209 heat shock protein HSP60 - Lyme disease spirochete 224-408 MZECPN60B MZECPN60B NID:

g309558 - Zea mays (strain B73)(library:Dash11 of P.S  
 189-373 CH60\_THEP3 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL  
 PROTEIN)(HEAT SHOCK 61 KD PRO 188-372 CH60\_STAEP 60 KD  
 CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(HEAT SHOCK  
 PROTEIN 6 189-373 CH60\_LACLA 60 KD CHAPERONIN (PROTEIN  
 CPN60)(GROEL PROTEIN). - LACTOCOCCUS LACTIS 188-374  
 CH61\_STRAL 60 KD CHAPERONIN 1 (PROTEIN CPN60 1)(GROEL  
 PROTEIN 1)(HSP58). - STRE 191-375 CH60\_CHLPN 60 KD  
 CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - CHLAMYDIA  
 PNEUMONI 224-408 MZECPN60A MZECPN60A NID: g309556 - Zea  
 mays (strain B73)(library:Dach 11 of P. 190-373 HECHSPAB1  
 HECHSPAB NID: g712829 - Helicobacter pylori  
 (individual isolate 85P) D 221-405 CH60\_ARATH  
 MITOCHONDRIAL CHAPERONIN HSP60 PRECURSOR. - ARABIDOPSIS  
 THALIANA (MOUS 224-408 CH60\_MAIZE MITOCHONDRIAL  
 CHAPERONIN HSP60 PRECURSOR. - ZEA MAYS (MAIZE). 190-374  
 CH60\_CHLTR 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL  
 PROTEIN)(57 KD CHLAMYDIAL HYP 189-373 CH60\_STAAU 60 KD  
 CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(HEAT SHOCK  
 PROTEIN 6 189-373 CH60\_CLOPE 60 KD CHAPERONIN (PROTEIN  
 CPN60)(GROEL PROTEIN). - CLOSTRIDIUM PERFRI 212-397  
 HS60\_YEAST HEAT SHOCK PROTEIN 60 PRECURSOR (STIMULATOR  
 FACTOR 1 66 KD COMPONENT) 217-403 CH60\_PYRSA 60 KD  
 CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - PYRENOMONAS  
 SALINA 191-377 CH60\_EHRCH 60 KD CHAPERONIN (PROTEIN  
 CPN60)(GROEL PROTEIN). - EHRLICHIA CHAFFEEN 191-375  
 CHTGROE1 CHTGROE NID: g144503 - C.trachomatis DNA. 188-  
 372 CH60\_THETH 60 KD CHAPERONIN(PROTEIN CPN60)(GROEL  
 PROTEIN). - THERMUS AQUATICUS 189-373 TAU294831 TAU29483  
 NID: g1122940 - Thermus aquaticus. 190-378 CH60\_RICTS 60  
 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN)(MAJOR  
 ANTIGEN 58)(5 189-375 SYCCPNC SYCCPNC NID: g1001102 -  
 Synechocystis sp. (strain PCC6803,) DNA.  
 190-373 CPU308211 CPU30821 NID: g1016083 - Cyanophora  
 paradoxa. 189-373 CH61\_MYCLE 60 KD CHAPERONIN 1 (PROTEIN  
 CPN60 1)(GROEL PROTEIN 1). - MYCOBACTERIU 239-423  
 PSU21139 PSU21139 NID: g806807 - pea. 191-377 CH60\_COWRU  
 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). -  
 COWDRIA RUMINANTIU 245-429 RUBB\_BRANA RUBISCO SUBUNIT  
 BINDING-PROTEIN BETA SUBUNIT PRECURSOR (60 KD CHAPERON  
 144-328 SCCPN60 SCCPN60 NID: g1167857 - rye.  
 153-338 CH60\_EHRRI 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL  
 PROTEIN)(55 KD MAJOR ANTIGEN) 245-429 RUBB\_ARATH RUBISCO  
 SUBUNIT BINDING-PROTEIN BETA SUBUNIT PRECURSOR (60 KD  
 CHAPERON 235-419 ATU49357 ATU49357 NID: g1223909 - thale  
 cress strain=ecotype Wassilewskija. 195-379 RUB1\_BRANA  
 RUBISCO SUBUNIT BINDING-PROTEIN ALPHA SUBUNIT (60 KD  
 CHAPERONIN ALPHA 189-374 CH62\_SYNY3 60 KD CHAPERONIN 2  
 (PROTEIN CPN60 2)(GROEL HOMOLOG 2). - SYNECHOCYSTI 178-  
 362 RUBA\_RICCO RUBISCO SUBUNIT BINDING-PROTEIN ALPHA  
 SUBUNIT (60 KD CHAPERONIN ALPHA 190-375 CH60\_ODOSI 60 KD  
 CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - ODONTELLA  
 SINENSIS 236-420 PSU21105 PSU21105 NID: g1185389 - pea.  
 224-409 CH60\_BRANA MITOCHONDRIAL CHAPERONIN CH60\_BACSU 60  
 KD CHAPERONIN (PROTEIN CPN60)(GROEL PROTEIN). - BACILLUS  
 SUBTILIS. 191-375 CH60\_AGRU 60 KD CHAPERONIN (PROTEIN  
 CPN60)(GROEL PROTEIN). - AGROBACTERIUM TUME 191-375

b36917 heat shock protein GroEL - Agrobacterium tumefaciens  
 191-375 PAU17072 PAU17072 NID: g576778 - Pseudomonas aeruginosa. 191-375 CH60\_RHILV 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - RHIZOBIUM LEGUMINO 187-373 CH61\_STRCO 60 KD CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL PROTEIN 1) (HSP58). - STRE 191-375 CH60\_COXBU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN B 191-375 CH62\_RHIME 60 KD CHAPERONIN B (PROTEIN CPN60 B) (GROEL PROTEIN B). - RHIZOBIUM ME 191-375 PSEGROESL1 PSEGROESL NID: g151241 - Pseudomonas aeruginosa (library: ATCC 27853) 189-372 CH61\_SYNY3 60 KD CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL HOMOLOG 1). - SYNECHOCYSTI 189-373 CH60\_CLOTM 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HSP-60). - CLOSTRIDI 191-373 CH60\_PSEPU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - PSEUDOMONAS PUTIDA 190-373 CH60\_SYNP7 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - SYNECHOCOCCUS SP. 190-374 CH60\_GALSU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - GALDIERIA SULPHURA 190-374 CH60\_ZYMMO 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - ZYMOMONAS MOBILIS. 191-375 JC2564 heat shock protein groEL - Zymomonas mobilis  
 191-375 CH60\_CHRVI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CHROMATIUM VINOSUM 189-373 CH60\_MYCTU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (65 KD ANTIGEN) (HEAT 191-375 CH60\_NEIME 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 189-373 CH60\_TREPA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (TPN60) (TP4 ANTIGEN) 190-374 CH60\_HELPY 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN 6 191-375 CH60\_NEIGO 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 222-406 CH61\_CUCMA MITOCHONDRIAL CHAPERONIN HSP60-1 PRECURSOR. - CUCURBITA MAXIMA (PUMPKI 189-373 CH60\_MYCPA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (65 KD ANTIGEN) (HEAT 230-414 MPU15989 MPU15989 NID: g559802 - Mycobacterium paratuberculosis. 224-408 S26582 chaperonin hsp60 - maize 191-375 S40247 heat-shock protein - Neisseria gonorrhoeae 189-373 CH60\_CLOAB 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CLOSTRIDIUM ACETOB 191-375 CH60\_NEIFL 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 190-373 CH60\_LEGPN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (58 KD COMMON ANTIGEN 222-406 CH62\_CUCMA MITOCHONDRIAL CHAPERONIN HSP60-2 PRECURSOR. - CUCURBITA MAXIMA (PUMPKI 191-375 CHTGROESL1 CHTGROESL NID: g402332 - Chlamydia trachomatis DNA. 64-248 S40172 S40172 NID: g251679 - Chlamydia psittaci pigeon strain P-1041. 189-373 SYOGROEL2 SYOGROEL2 NID: g562270 - Synechococcus vulcanus DNA. 191-375 CH60\_CHLPS 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (57 KD CHLAMYDIAL HYP 188-372 CH62\_STRAL 60 KD CHAPERONIN 2 (PROTEIN CPN60 2) (GROEL PROTEIN 2) (HSP56). - STRE 189-373 CH62\_MYCLE 60 KD CHAPERONIN 2 (PROTEIN CPN60 2) (GROEL PROTEIN 2) (65 KD ANTIGEN) 236-420 MSGANTM MSGANTM NID: g149923 - M.leprae DNA, clone Y3178.



CPN60 PRECURSOR. - BRASSICA NAPUS (RAPE). 105-289 PMSARG2  
PMSARG2 NID: g607157 - Prochlorococcus marinus.  
234-417 RUB2\_BRANA RUBISCO SUBUNIT BINDING-PROTEIN ALPHA  
SUBUNIT PRECURSOR (60 KD CHAPERON 75-259 CRECPN1A CRECPN1A  
NID: g603910 - Chlamydomonas reinhardtii cDNA to mRNA.  
215-400 P60\_CRIGR MITOCHONDRIAL MATRIX PROTEIN P1  
PRECURSOR (P60 LYMPHOCYTE PROTEIN)(CH224-408 CRECPN1B  
CRECPN1B NID: g603912 - Chlamydomonas reinhardtii cDNA to  
mRNA. 191-375 RUBA\_WHEAT RUBISCO SUBUNIT BINDING-PROTEIN  
ALPHA SUBUNIT PRECURSOR (60 KD CHAPERON 189-373 B47292  
heat shock protein groEL - Mycobacterium tuberculosis  
206-391 CELHSP60CP CELHSP60CP NID: g533166 -  
Caenorhabditis elegans (strain CB1392) cDNA 215-400  
P60\_HUMAN MITOCHONDRIAL MATRIX PROTEIN P1 PRECURSOR (P60  
LYMPHOCYTE PROTEIN)(CH 215-400 P60\_MOUSE MITOCHONDRIAL  
MATRIX PROTEIN P1 PRECURSOR (P60 LYMPHOCYTE PROTEIN)(CH  
215-400 P60\_RAT MITOCHONDRIAL MATRIX PROTEIN P1 PRECURSOR  
(P60 LYMPHOCYTE PROTEIN)(CH 215-400 A41931 chaperonin  
hsp60 - mouse  
197-382 MMHSP60A MMHSP60A NID: g51451 - house mouse. 218-  
402 CH63\_HELVI 63 KD CHAPERONIN PRECURSOR (P63). -  
HELIOTHIS VIRESCENS (NOCTUID MOTH) 205-390 EGHSP60GN  
EGHSP60GN NID: g1217625 - Euglena gracilis. 222-407  
HS60\_SCHPO PROBABLE HEAT SHOCK PROTEIN 60 PRECURSOR. -  
SCHIZOSACCHAROMYCES POMBE 198-385 S61295 heat shock  
protein 60 - Trypanosoma cruzi  
198-385 TRBMTHSP TRBMTHSP NID: g903883 - Mitochondrion  
Trypanosoma brucei (strain EATRO 8-69 ECOGROELA ECOGROELA  
NID: g146268 - E.coli DNA, clone E. 142-325 ENHCPN60P  
ENHCPN60P NID: g675513 - Entamoeba histolytica (strain  
HM-1:IMSS) DNA. 257-433 CH60\_PLAFG MITOCHONDRIAL  
CHAPERONIN CPN60 PRECURSOR. - PLASMODIUM FALCIPARUM (ISO  
1-90 CRECPN1C CRECPN1C NID: g603914 - Chlamydomonas  
reinhardtii cDNA to mRNA.  
5-65 ATTS0779 ATTS0779 NID: g17503 - thale cress.  
189-373 CH60\_MYCGE 60 KD CHAPERONIN (PROTEIN CPN60)(GROEL  
PROTEIN). - MYCOPLASMA GENITAL 228-411 HTOHSP60X  
HTOHSP60X NID: g553068 - Histoplasma capsulatum (strain  
G217B) DNA. 190-297 CH60\_SYN6 60 KD CHAPERONIN (PROTEIN  
CPN60)(GROEL PROTEIN)(FRAGMENT). - SYNECHO 169-245  
RUBA\_ARATH RUBISCO SUBUNIT BINDING-PROTEIN ALPHA SUBUNIT  
(60 KD CHAPERONIN ALPHA

The polypeptide may further comprise a polyamino acid sequence, preferably an N-terminal polyamino acid sequence although a C-terminal sequence may be present instead or in addition to an N-terminal sequence. The  
5 polyamino acid sequence may be selected from the same or different amino acid residues. When the same amino acid residue is repeated a particularly preferred polyamino acid sequence is a polyhistidine sequence.

10 Whether composed of the same or different amino acid residues, the further polyamino acid sequence may comprise any number of amino acid residues so long as chaperonin activity is provided. The other amino acid  
15 acid sequence could be selected from any of the twenty or so essential amino acids common to biological systems or of any amino acid variant or derivative. When the polyamino acid sequence is other than a homopolymer then it may comprise a repeated sequence of two or more amino  
20 acid residues. The amino acid sequence may encode a portion of another known protein or polypeptide or it may even be random.

The further polyamino acid sequence preferably also  
25 includes a cleavage site cleavable by a cleavage agent. A preferred cleavage agent is thrombin although any other suitable agent will suffice. The sequence of amino acid residues is of course selected to permit cleavage by the desired cleavage agent.

30 The further polyamino acid sequence preferably comprises 17 to 39 amino acids although more than 39 and less than 17 amino acids may also be employed.

The polypeptide may be attached to a support, preferably in immobilised form, optionally immobilised to a chromatographic matrix, more preferably an agarose resin. When an agarose resin is used it is preferably a nickel-  
5 nitrilo-tri-acetic acid (NTA)-ligated agarose resin. This has affinity for a polypeptide having a polyhistidine tail.

The polypeptide of the invention may be obtained by  
10 recombinant means. Alternatively, the polypeptide may be produced by a routine chemical synthesis using standard polypeptide synthesis procedures known in the art. If produced by recombinant means the polypeptide may be fused to a heterologous protein or polypeptide.

15

The inventors have found that the fragments with polyhistidine tails of 17 amino acid residues sht-GroEL191-345 and sht-GroEL191-376 as well as GroEL191-345  
20 are potent facilitators of the folding of inactive forms of cyclophilin A and rhodanese, and catalyse the unfolding of barnase. Maximal refolding yield was obtained at stoichiometric concentrations of cyclophilin A and apical domain, indicating the formation of a 1:1  
25 complex between chaperone fragment and substrate protein during refolding. The fragments sht-GroEL191-345 and sht-GroEL191-376 when attached to nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin are also active in enhancing the folding yield of destabilised mutants of  
30 indole glycerol-phosphate synthase and of the protein cyclophilin A. This proves the monomer has refolding activity and also demonstrates that the immobilised protein is functionally useful.

Fragments of GroEL may be produced either from recombinant DNA methods or from protein chemistry or any means of chemical synthesis, or the equivalent fragments of homologous hsp60 (cpn60) proteins or any natural  
5 variants or any variants produced by mutagenesis, or of larger fragments containing the sequences 191-376, 191-345 of GroEL from *E. coli*.

In tenth aspect the invention provides a nucleic acid  
10 molecule comprising a nucleotide sequence encoding a polypeptide of the invention, or a nucleotide sequence hybridisable thereto and optionally encoding a polypeptide having chaperone activity.

15 Preferred characteristics of the nucleotide sequence correspond to the preferred features of the polypeptides of the aspects of the invention as hereinbefore defined.

This aspect of the invention therefore includes a  
20 recombinant DNA molecule for use in cloning and/or expressing a DNA sequence, said recombinant DNA molecule comprising:

- 25 (a) a nucleotide sequence encoding amino acid residues 191-376 of GroEL,
- (b) a nucleotide sequence encoding amino acid residues 191-345 of GroEL,
- 30 (c) a nucleotide sequence encoding amino acid residues 193- 337,
- (d) a nucleotide sequence encoding amino acid residues of GroEL selected from amino acid residues:

- (i) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336, 191-337, 191-338, 191-339, 191-340, 191-341, 191-342, 191-343, 191-344, 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352, 191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360, 191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368, 191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-376, or
- (ii) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336, 192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344, 192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352, 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360, 192-361, 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368, 192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-376, or
- (iii) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336, 193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344, 193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352, 193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360, 193-361, 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368, 193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-376.
- (e) 230-271, 229-271, 229-272, 228-272, 228-273, ...et seq... 194-328, 194-329, or

(f) a nucleotide sequence hybridisable to any of (a), (b), (c), (d) or (e) above and encoding a monomeric polypeptide having chaperone activity, or

- 5 (g) degenerate nucleotide sequences corresponding to (a), (b), (c), (d), (e) or (f) above.

Further features of the nucleic acid sequence may be as described hereinbefore in relation to the polypeptides of  
10 the invention; the nucleic acid sequence having the appropriate nucleotide sequence which on expression provides a polypeptide which possesses these features.

In eleventh aspect the invention provides a vector  
15 comprising a nucleic acid as hereinbefore defined.

In twelfth aspect the invention provides a host cell transformed with a vector or a nucleic acid molecule as hereinbefore defined.

20

In thirteenth aspect there is provided a method of making a polypeptide of the invention comprising transforming a host cell with a nucleic acid encoding said polypeptide, culturing the transformed cell for and expressing said  
25 polypeptide. Expression may be direct or as a fusion product. When the polypeptide product is expressed as a fusion then the method preferably includes a step wherein the expressed polypeptide product is subject to cleavage. The polypeptide may be recovered from the  
30 transformed cells expressing it.

In fourteenth aspect the invention provides a pharmaceutical formulation comprising a polypeptide of

the invention, optionally together with a diluent, carrier or excipient.

- 5 The active ingredients of a pharmaceutical composition comprising the polypeptide are contemplated to exhibit excellent therapeutic activity, for example, in the alleviation of Alzheimer's disease when administered in amount which depends on the particular case. Dosage regima may be adjusted to provide the optimum therapeutic
- 10 response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.
- 15 The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of
- 20 administration, the active ingredient may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredient.
- 25 In order to administer the polypeptide by other than parenteral administration, it will be coated by, or administered with, a material to prevent its inactivation. For example, the polypeptide may be administered in an adjuvant, co-administered with enzyme
- 30 inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as

polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin.

Liposomes include water-in-oil-in-water CGF emulsions as  
5 well as conventional liposomes.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures  
10 thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use  
15 include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability  
20 exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol  
25 (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle  
30 size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal



agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption  
5 of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

10 Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are  
15 prepared by incorporating the sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable  
20 solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

25 When the polypeptide is suitably protected as described above, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin  
30 capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules,

elixirs, suspensions, syrups, wafers, and the like. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained.

5

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; 10 a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit 15 form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit.

20 For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of 25 course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

30

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like.

The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such as active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In ninth aspect there is provided polypeptide of the invention as hereinbefore defined for use in the

treatment of disease. Consequently there is provided the use of a polypeptide of the invention for the manufacture of a medicament for the treatment of disease associated with aberrant protein/polypeptide structure. The

5 aberrant nature of the protein/polypeptide may be due to misfolding or unfolding which in turn may be due to an anomalous eg mutated amino acid sequence. The protein/polypeptide may be destabilised or deposited as plaques eg as in Alzheimer's disease. The disease might

10 be caused by a prion. A polypeptide-based medicament of the invention would act to renature or resolubilise aberrant, defective or deposited proteins.

In fifteenth aspect there is provided a nucleic acid

15 molecule in accordance with other aspects of the invention for use in the treatment of disease. Consequently, there is provided the use of a nucleic acid molecule of the invention for the manufacture of a medicament for the treatment of disease associated with

20 protein/polypeptide structure. Genetic therapy *in vivo* is therefore provided for by way of introduction and expression of DNA encoding the monomeric polypeptide in cells/tissues of an individual to provide chaperonin activity in those cells/tissues.

25 In sixteenth aspect there is provided the use of a polypeptide of the invention for altering the structure of a molecule, particularly a protein or polypeptide and the alteration in structure may be by folding, unfolding

30 or resolubilising. Preferably, the stoichiometry between the monomeric polypeptide of the invention and the molecule being altered is about 1:1, preferably 1:1.

In seventeenth aspect there is provided a method of reconditioning a molecule preferably a protein comprising contacting said protein with a polypeptide of the invention. Preferably the protein is subjected to  
5 inactivation or denaturation prior to contacting with said polypeptide. The polypeptide may be immobilised to a solid phase, preferably a chromatographic matrix, and the contacting of protein and polypeptide is carried out by applying the protein to the top of a bed of the matrix  
10 packed in a column and then eluting the polypeptide through the column.

This aspect of the invention therefore provides for the use of a polypeptide as hereinbefore defined for altering  
15 the structure of a molecule, preferably a protein or polypeptide and the alteration in structure is by folding, unfolding or refolding. The stoichiometry between the polypeptide and the molecule being altered may be about 1:1.

20

In nineteenth aspect the invention provides for the use of a polypeptide of the invention the purification or increase in yield, specific activity or quality of biological molecules, preferably said polypeptide being  
25 attached to a support.

In twentieth aspect the invention provides a kit for reconditioning or refolding a molecule, preferably a protein, comprising a polypeptide as hereinbefore defined  
30 immobilised to a solid phase and a container for holding said solid phase polypeptide.

In twenty-first aspect the invention provides for the use of a polypeptide as hereinbefore defined in the

production of a protein or polypeptide by recombinant means, wherein the said polypeptide is co-expressed with the protein or polypeptide thereby to improve the yield or quality of the protein or polypeptide.

5

In twenty-second aspect the invention provides an antibody reactive against a polypeptide of the invention. The invention also provides for the use of the antibody in the treatment of disease. Also, the invention  
10 provides for the use of the antibody in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure.

In twenty-third aspect the invention provides various  
15 methods of treating disease, namely a method of treating disease in which an effective amount of a polypeptide of the invention is administered. There is also a method of treating disease which comprises administering an effective amount of an inhibitor of the chaperone  
20 activity of a polypeptide of the invention. Preferably, said inhibitor is an antibody. Also, there is a method of treating disease by gene therapy which utilises a construct encoding a polypeptide of the invention or an antagonist thereof.

25

The monomeric apical domain of GroEL or its fragments catalyse amide proton exchange of barnase, and facilitate refolding of rhodanese and cyclophilin A in the absence of cofactors. Thus, GroEL has an intrinsic chaperone  
30 activity, which is not restricted to its oligomeric state or central cavity. The C-terminal  $\alpha$ -helices of the apical domain are not directly involved in polypeptide binding, and form a separate folding unit. At physiological temperature, about 50% of the C-terminus is

physiological temperature, about 50% of the C-terminus is unfolded, allowing high mobility movements of the polypeptide binding "domain core". Flexibility within the apical domain may be crucial for cooperative binding of a wide range of proteins with different native structure, and for the conformation change of GroEL on binding to the co-chaperonin GroES. The crystal structure of the "domain core" of the apical domain has an overall B-factor 60% lower than that of the same region in intact GroEL. The overall fold of the fragment is similar to the corresponding region of intact GroEL, but the amount of secondary structure is considerably larger. There are three  $3_{10}$ -helices in the structure of the fragment, which are involved in binding of polypeptide and/or GroES.

The inventors have expressed the apical domain of GroEL as a stable monomeric protein and with high yield in *E. coli*, allowing its activity, folding, and structure to be studied separately from those of the equatorial and intermediate domains. The isolated, apical domain is functional in polypeptide binding (Figure 2), and it facilitates protein folding even when truncated to remove its C-terminal  $\alpha$ -helices, H11 and H12 (Figure 3). The apical domain slows down spontaneous refolding of rhodanese and cyclophilin A by >15-fold and >150-fold, respectively (Figures 3c-e). The inventors have shown increased refolding yield of rhodanese and cyclophilin A in the presence of apical domain (Figure 3), which is too small to reassociate and to form a cavity, clearly demonstrates the presence of an intrinsic chaperone activity in GroEL, which is independent of a central cavity. This is in agreement with NMR experiments on barnase in the presence of apical domain (Figure 2). The

presence of micromolar concentrations of GroEL considerably broadens the resonance lines of barnase owing to a fast association and idssociation of native barnase from the slowly rotational tumbling GroEL (Zahn et al (1996) Science 271: 642-645). If the apical domain and barnase would form a complex of large molecular size, i.e. containing seven or 14 molecules of apical domain, one would expect a considerable degree of line broadening in the NMR spectra of barnase in the presence of apical domain. However, this is not what was observed, even not at eight-fold higher monomer concentration of apical domain than of GroEL (Zahn et al (1996) Science 271: 642-645). The complex between apical domain and barnase, therefore, rather seems to be of low molecular weight and probably has a 1:1 stoichiometry, which would be consistent with the binding stoichiometry of the apical domain and cyclophilin A (Figure 3e).

The intrinsic chaperone activity of monomeric apical domain facilitates refolding of rhodanese even though physiological conditions, GroES, and ATP are required in the presence of intact GroEL. Although the inventors do not wish to be bound by any particular hypothesis the role of GroES appears to be to weaken the affinity of GroEL for substrates, and to prevent premature dissociation of aggregation prone states: there has to be a balance between tight binding for the annealing activity and weaker binding to allow folding (Corrales F J and Fersht A R (1996) Proc Natl Acad Sci USA 93: 4509-4512). The weaker binding of rhodanese to the fragment must be adequate for chaperoning activity and weak enough for folding. This is in agreement with the observed similar rate constant for refolding of rhodanese in the presence of apical domain and in the presence of GroEL,



GroES, and ATP (Figures 3b-d), indicating a similar binding affinity. The complex structure of GroEL, and the modulation of its substrate affinity by GroES and nucleotides, must be to allow the efficient folding of a wide range of proteins, with a wide variation of affinities for GroEL. The mechanism, in general, may involve several components: GroES binds to the *cis*-end of a GroEL-substrate complex and displaces the bound polypeptide into the cavity; the dissociated and encapsulated polypeptide chain then refolds in an enlarged folding cavity to a native-like state, until it is released upon the dissociation of GroES; and, the cycle of binding and dissociation of unfolded protein, GroES, ATP, and GroEL may be repeated several times, before the substrate protein can adopt a native conformation (Hunt et al (1996) *Nature* 379: 37-45; Weissman et al (1996) *Cell* 84: 481-490; Weissman J S et al (1995) *Cell* 83: 577-587; Mayhew M et al (1996) *Nature* 379: 420-426; Corrales F J and Fersht A R (1996) *Proc Natl Acad Sci USA* 93: 4509-4512). Polypeptides with intermediate affinity for GroEL, such as cyclophilin A (Figure 3e), bind transiently to GroEL and folding is facilitated without the need for co-factors.

Helices H11 and H12 are much less stable than the "domain core" of the apical domain, and form a separate folding unit (Figure 4). At physiological temperature, about 50% of the secondary structure of the low-melting helices is unfolded and thus becomes flexible. But, *in vivo*, the C-terminus is not degraded, implying that the primary structure is not accessible to degradation by *E. coli* proteases. The low stability of the C-terminal helices allows movements and larger fluctuations of the "domain cores" within the GroEL-ring. This flexible arrangement

of the apical domain may be implicated in the cooperative binding of unfolded polypeptide to the seven subunits of GroEL in each ring. It is understandable that flexibility is required for binding to a large variety of proteins with different amino acid sequence, and secondary and tertiary structure. The observed low stability of the C-terminus may also contribute to the large conformational change in GroEL on binding of GroES, which has been suggested to occur through rigid body movement through the  $\beta$ -sheet hinge region of the intermediate domain (Braig K et al (1994) Nature 371: 578-586).

The production and crystallisation of monomeric fragments of the apical domain by the inventors has allowed a determination of the three-dimensional structure of the polypeptide binding part of GroEL with B-factors much lower than those of the equivalent regions in GroEL (Figure 5) (Braig K et al (1994) Nature 371: 578-586; Braig et al (1995) Nature Struct Biol 2: 1083-1094; Boisvert et al (1996) Nature Structure Biology 3: 170-177), perhaps because of favourable packing interactions within the crystal, or the absence of the flexible C-termini in the GroEL fragment, or a combination of both. Particularly interesting is the existence of  $3_{10}$ -helices within the apical domain. These tightly packed helices are much less common than  $\alpha$ -helices, and the majority are very short, with 96% being less than four residues. They are usually found at the end of  $\alpha$ -helices, where the final turn may have this conformation, or between two  $\beta$ -strands. All the  $3_{10}$ -helices of the apical domain are localised within regions, which have been shown to be involved in polypeptide and/or GroES binding (Fenton et al (1994) Nature 371: 614-619). The third  $3_{10}$ -helix has

an unusual length of 10 residues. A  $3_{10}$ -helix with a similar length (9 residues) has been found in aconitase (Robbins et al (1989) Proteins: Structure, Function and Genetics 5: 289-312), an enzyme of the Calvin cycle.

- 5 Interestingly, the apical domain and aconitase also share a similar fold: a central  $\beta$ -sheet linked by  $\alpha$ -helices, suggesting that they may use a common mechanism for substrate binding.
- 10 Preferred embodiments of the invention will now be described in detail by way of the following examples and with reference to the accompanying drawings in which:

Figure 1 shows the amino acid sequence of the apical  
15 region of GroEL from *E. coli* including further amino acid residues providing an N-terminal histidine-tail (ht).

Figure 2a is a bar chart showing the catalytic effect of GroEL 191-345 on the amide proton exchange of unfolded  
20 barnase. The ratio of the protection factors of the 39 measurable amide protons of barnase in the absence (P) and presence (P+(G)) of the GroEL fragment is shown.

Figure 2b is a bar chart similar to that of Figure 2a but  
25 for the GroEL fragment 191-345 with a 17 residue N-terminal histidine tail (sht-GroEL 191-345).

Figure 2c is a bar chart similar to that of Figure 2a but  
for the GroEL fragment 191-376 with a 17 residue N-  
30 terminal histidine tail (sht-GroEL 191-376).

Figure 2d is a plot of observed exchange rate constants in the absence of chaperonin ( $k_{ex}^{obs}$ ) against those in the

presence of chaperonin ( $k_{ex}^{obs}(+G)$ ) for GroEL fragment 191-345. Global, mixed and local amide protons are displayed by circles, triangles and squares respectively.

- 5 Figure 2e is a plot similar to that of Figure 2d but for sht-GroEL 191-345.

Figure 2f is a plot similar to that of Figure 2d but for sht-GroEL 191-376.

10

Figure 3a is a bar chart and table showing the relative enzymatic activity of rhodanese after refolding in the presence or absence of GroEL, GroES, ATP, sht-GroEL 191-345, sht-GroEL 191-376 or bovine serum albumin (BSA).

15

Figure 3b is a plot showing the refolding kinetics of rhodanese in the presence of GroEL, GroES and ATP.

- 20 Figure 3c is a plot showing the refolding kinetics of rhodanese in the presence of various concentrations of sht-GroEL 191-345.

- 25 Figure 3d is a plot showing the refolding kinetics of rhodanese in the presence of various concentrations of sht-GroEL 191-376.

- Figure 3e is a plot comparing the refolding kinetics of specified concentrations of sht-GroEL 191-376, sht-GroEL 191-345 and GroEL.

30

Figure 4a is a trace of the thermal denaturation of sht-GroEL 191-376 (upper trace) and sht-GroEL 191-345 (lower trace) monitored by far ultra violet-circular dichroism at 222nm.

Figure 4b is a trace as in Figure 4a but thermal denaturation is monitored by differential scanning calorimetry.

5

Figure 5a shows a three dimensional representation of the structure of sht-GroEL 191-345.

10 Figure 5b shows a three dimensional representation of the backbone structure of sht-GroEL 191-345.

Figure 5c shows a three dimensional representation of electron densities of sht-GroEL 191-345 viewed along the helices H8 and H9.

15

Figure 6 is a trace showing the elution of denatured mutant IGPS from a column of immobilised GroEL 191-345 developed with refolding buffer.

20 Figure 7 shows the amino acid sequence of GroEL from *E. coli* numbered from the N-terminal methionine.

Figure 8 shows the amino acid sequence of a portion of GroEL wherein the residues involved in binding peptides  
25 are highlighted.

Figures 9a-c comprise partial amino acid sequences of known cpn 60 family members aligned with one another for comparison.

30

Example 1 - Cloning and expression of the apical domain of GroEL and various of its fragments

The apical domain of GroEL (GroEL 191-376) and various C-terminally truncated fragments of the apical domain were cloned by polymerase chain reaction (PCR) into the polylinker site of a pRSET A vector (Invitrogen), coding for an N-terminal histidine-tail, which contained an engineered thrombin cleavage site. The histidine-tail was composed of 36 amino acids (Invitrogen) or 17 amino acids. For the PCR reaction, the plasmid pOF39 (Fayet O et al (1989) J Bacteriol 171: 1379-1385) was used as a template together with two primers flanking the respective GroEL fragment with BamHI and ClaI restriction enzyme sites. This permitted the cloning of the PCR fragment into the polylinker of pRSET A. PCR was performed with Pfu (Stratagene) to reduce the risk of undesired random mutations. The reaction was performed in a volume of 25 µl for 25 cycles with 400 nM of primer and 200 µM of deoxynucleoside-5'-triphosphates. The annealing temperature was 65°C. The following primers were used for the PCT to generate DNA encoding the apical region of GroEL and a variety of fragments thereof: 5' flanking: 5'-CGG ATC CGA AGG TAT GCA GTT CGA CCG; 3' flanking (s)ht-GroEL191-376, 5'-CGA ATT CTT AAA CGC CGC CTG CCA GTT TCG; 3' flanking (s)ht-GroEL191-345, 5'-CGA ATT CTT AAC GGC CCT GGA TTG CAG CTT C; 3' flanking ht-GroEL191-337, 5'-CGA ATT CTT AAC CCA CGC CAT CGA TGA TAG TGG TG; 3' flanking ht-GroEL191-328, 5'-CGA ATT CTT AGT CTT TGT TGA TCA CAA CAC GTT TAG CCT GAC; 3' flanking ht-GroEL191-322, 5'-CGA ATT CTT AAC GTT TAG CCT GAC CCA GGT CTT CCA; 3' flanking ht-GroEL191-298, 5'-CGA ATT CTT AAC CGC CAG TCA GGG TTG CGA TAT C.

The following protocol was used for expression and purification of the fragments of GroEL in *E. coli* TG2 cells. With reference to Figure 1, the expression vector

used coded for an N-terminal histidine-tail (ht) composed of 36 amino acids and containing a thrombin cleavage site (vertical arrow). Alternatively, a shorter version of this histidine-tail (sht) containing 17 amino acids was used. The N- and C-terminal ends of the generated fusion proteins, namely ht-GroEL 191-298, ht-GroEL 191-322, ht-GroEL 191-328, ht-GroEL 191-337, ht-GroEL 191-345, ht-GroEL 191-376, sht-GroEL 191-345, and sht-GroEL 191-376 are indicated by rectangular arrows.

10

Two litres of L-Broth medium plus ampicillin was inoculated 1:100 with an over-night culture of TG2 cells containing the respective plasmid. At an  $A_{600}$  of 0.3, expression was induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside to 0.2 mM final concentration and M13/T7-phage at a multiplicity of infection of 10 phages per cell. The cells were harvested 8 h after induction, centrifuged, and resuspended in 200 ml buffer A (50 mM Tris-HCl pH 8.2, 300 mM NaCl).

20

After sonication and centrifugation, the soluble protein fraction was added to 20 ml nickel-NTA agarose resin and stirred for 10 min. The resin was washed with 200 ml buffer A, and the histidine-tail containing fusion protein was eluted with 50 ml buffer A containing 200 mM imidazole. The eluted protein was precipitated with 80% ammonium sulfate, re-dissolved in 4 ml of buffer B (50 mM Tris-HCl pH 8.2, 150 mM sodium chloride), and was loaded on a HiLoad 26/60 Superdex 75 column (Pharmacia), which was equilibrated with buffer B. The fragment GroEL 191-345 was produced by thrombin-cleavage of ht-GroEL 191-376 before gel filtration. The cleaving reaction was carried out for several days in buffer B after addition of 250  $\mu$ l thrombin (Sigma, 1U/ $\mu$ l) to the protein solution eluted

30

from the nickel-NTA column. The GroEL fragments were analyzed by quantitative amino acid analysis, N-terminal sequencing, and mass spectrometry.

- 5 In Figure 1, secondary structure is indicated by boxes and arrows for  $\alpha$ -helix (grey) or  $3_{10}$ -helix (white) and  $\beta$ -sheet structure, respectively. Assignment of secondary structure of residues 191 to 336 was done on the basis of the crystal structure of sht-GroEL 191-345 (Table 1 and  
10 Fig 5) using PROCHECK (Laskowski R A et al (1993) J Appl Cryst 26: 283-291) and the algorithm of Kabsch & Cander (Kabsch W and Sander C (1983) Biopolymers 22: 2577-2637). Numbering of  $\alpha$ -helices and secondary assignment of residues 337 to 376 according to Braig et al (Braig K et  
15 al (1995) Nature Struct Biol 2: 1083-1094).

- Functional apical domain of GroEL and various functional fragments of the apical domain in *E. coli* were expressed allowing the study of chaperone activity, folding, and  
20 crystal structure of the polypeptide binding part of GroEL. In particular, the apical domain of GroEL (GroEL 191-376) and various fragments of the apical domain (Fig 1) were expressed in *E. coli* as fusion proteins containing an N-terminal histidine-tail, and this allowed  
25 for a straightforward purification using a nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin. The histidine-tail of either 39 (ht) or 17 amino acids (sht) contained a sequence of six histidine residues and a thrombin cleavage site. The apical domain was expressed  
30 at >20 mg purified protein per litre of culture as were the smaller fragments lacking the C-terminal  $\alpha$ -helices, H11 and H12. Further truncation before residue 329 led to considerable destabilisation of the apical domain.



The fragment GroEL 191-345, containing no histidine-tail and without the two helices was generated by thrombin cleavage of purified ht-GroEL 191-376.

5 The apical region and monomeric fragments were found to have chaperone activity. The circular dichroism (CD) of sht-GroEL 191-376, sht-GroEL 191-345, and GroEL 191-345 in the far ultra violet (UV) region and in the near UV  
10 structure, respectively. The apical domain and the fragment truncated at position 345 are monomeric at micromolar concentrations, when determined by ultracentrifugation. However, the line widths in the nuclear magnetic resonance (NMR) spectra of GroEL 191-345  
15 are larger than expected for a 17 kD protein but small than for a stable dimer (data not shown), indicating a fast intermolecular interaction between monomers on the NMR time scale (Wüthrich, K, NMR of Proteins and Nucleic Acids (Wiley, New York, 1986). There seems to be a low  
20 affinity self-recognition of the apical domain.

Example 2 - Binding of GroEL 191-345 , sht-GroEL 191-345, and sht-GroEL 191-376 to unfolded barnase

25 Exchange experiments were carried out at 33°C in 20 mM imidazole buffer as described in Zahn et al (1996) Science 271: 642-645. The concentration of barnase was 2.4 mM. The results are shown in Figures 2a, 2b and 2c; locally and globally exchanging amide protons, and amide  
30 protons exchanging by a mixture of both mechanisms (Perrett et al (1995) Biochemistry 34: 9288-9298 and Clarke et al (1993) Proc Natl Acad Sci USA 90: 9837-9841) are indicated by white, black, and grey bars,

respectively. The indole NH proton of Trp71 is indicated by 71s.

The same experiments are shown in Figures 2d, 2e and 2f respectively. The data in Figure 2d is fitted to  $k_{\text{ex}}^{\text{obs}}(+G) = Ck_{\text{ex}}^{\text{obs}}$ , where  $C$  is a factor by which EX2 mechanism exchange is catalysed by GroEL fragment. The values of  $C$  for the globally, locally, and mixed amide protons of barnase are 10, 5, and 1. The data in Figures 2e and 2f is fitted to  $k_{\text{ex}}^{\text{obs}}(+G) = Ck_{\text{ex}}^{\text{obs}} + k_{\text{ex}}^{\text{obs}}(\text{G.U.})$ , where  $k_{\text{ex}}^{\text{obs}}(\text{G.U.})$  is the observed EX1 rate constant for global exchange of barnase in the presence of GroEL fragment. Figures 2d, 2e and 2f serve to demonstrate a mechanism of amide proton exchange of barnase. In Fig 2a and 2d there is only an estimate of the final concentration of GroEL fragment. At the high initial protein concentration used, GroEL 191-345 tended to crystallise during the exchange experiment.

Figures 2a, 2b and 2c show that the GroEL fragments catalyse, under folding conditions, exchange of amide protons of native barnase that are known to exchange only from its fully unfolded state (Perrett et al (1995) Biochemistry 34: 9288-9298). Thus, like intact GroEL (Zahn et al (1996) Science 271: 642-645), the apical domain binds with high affinity to unfolded barnase, and helices H11 and H12 are not essential for polypeptide binding (Figures 2a and 2b). The presence of the N-terminal histidine-tail did not abolish binding activity (Figures 2b and 2c).

30

The mechanism of exchange at pD 6.6 is EX2 (Figure 2d), but changes to EX1 at higher pD (Figures 2e and 2f). In an EX2 mechanism (Hvidt A and Nielsen S O (1966) Advan

Protein Chem 21: 287-386), the observed rate constant is limited by the intrinsic rate constant for exchange, which depends on pD. The criterion for an EX2 mechanism is that the rate constant for reprotection (ie a  
5 refolding or dissociation reaction) is much greater than the intrinsic rate constant. At the other extreme, when intrinsic exchange is much faster than reprotection, the mechanism becomes EX1 (Hvidt A and Nielsen S O (1966) *supra*) and the observed rate constant is simply equal to  
10 the rate constant for the formation of the unprotected state. Thus, from the exchange behaviour (and assuming that the presence of the histidine-tail does not effect binding) the rate constant for dissociation of barnase from the apical domain is about  $2 \text{ s}^{-1}$ , which is less than  
15 five-fold larger than that from intact GroEL (Zahn et al (1996) *supra*).

### Example 3 - Refolding of rhodanese

20 Rhodanese refolding assays were performed using GroEL, GroES, ATP, sht-GroEL 191-345 and sht-GroEL 191-376 and carried out as described by Horowitz (Horwitz P M in Protein Stability and Folding (eds Shirley B A) 361-368 (Humana Press, 1995)).

25 In more detail, rhodanese (9  $\mu\text{M}$ ) was unfolded for 45 min at  $25^\circ\text{C}$  in the presence of 8 M urea and 1 mM  $\beta$ -mercaptoethanol. Refolding was initiated by diluting 3  $\mu\text{l}$  of the unfolded rhodanese into a final volume of 250  
30  $\mu\text{l}$  of a standard buffer containing 50 mM Tris-HCl pH 7.8, 50 mM sodium thiosulphate, 10 mM  $\text{MgCl}_2$ , 10 mM KCl. GroEL, GroES, ATP, apical domain (or its C-truncated form) and bovine serum albumin (BSA) were included as indicated, at final concentrations of 2.5  $\mu\text{M}$  monomer, 2.5  $\mu\text{M}$  monomer,

2mM, 2.5  $\mu$ M and 45  $\mu$ g/ml (the same concentration by weights as the GroEL fragments), respectively. After incubation for 50 min at 25°C, rhodanese activity was measured by adding 25  $\mu$ l from the refolding mixture to 1 ml of 50 mM  $\text{Na}_2\text{S}_2\text{O}_3$ , 50 mM KCN and 40 mM potassium phosphate buffer pH 8.6. The reaction was terminated after 15 min incubation by addition of 0.5 ml of 18% formaldehyde. Colour was developed by mixing with 1.5 ml of ferric nitrate reagent (400 g  $\text{FeNO}_3 \cdot 9\text{H}_2\text{O}$ ; 800 ml 65%  $\text{HNO}_3$  in a final volume of 3 dm<sup>3</sup>) prepared as indicated (Perrett et al (1995) *supra*).

Figure 3a shows the relative enzymatic activity of rhodanese (0.1  $\mu$ M) after refolding in the presence (+) or absence (-) of GroEL (2.5  $\mu$ M monomer), GroES (2.5  $\mu$ M monomer), ATP (2 mM), sht-GroEL 191-345 (2.5  $\mu$ M), sht-GroEL 191-376 (2.5  $\mu$ M), or bovine serum albumin (BSA; 45  $\mu$ g/ml), from 8 M urea (U). 100% activity was obtained with native rhodanese (N) alone.

Figure 3b shows the refolding kinetics of rhodanese in presence GroEL, GroES, and ATP (•). The final concentrations are the same as in Figure 2a. 100% activity was obtained with native rhodanese (O).

Figures 3c and 3d show the refolding kinetics of rhodanese in the presence of 0.18  $\mu$ M (□), 2.5  $\mu$ M (•), or 5  $\mu$ M (O) sht-GroEL 191-345 and sht-GroEL 191-376, respectively.

Figure 3a shows a rhodanese refolding activity of about 42.5% for shtGroEL 191-345 which is about 37.5% above background refolding as shown by the control of unfolded rhodanese (U) alone.

The results of Figure 3b are for comparative purposes and show a time course of refolding activity for GroEL, GroES and ATP.

5

Figure 3c shows the rhodanese refolding kinetics at various concentrations of sht-GroEL 191-345. Refolding activities of about 50% are achieved at about 25 mins.

10 Figure 3d is similar to Figure 3c but for sht-GroEL 191-376 and showing refolding activities of about 40% after 25 mins.

Enzymatic activity was obtained from the absorbance at  
15 460 nm of the complex formed between thiocyanate and ferric ion. Results correspond to the average of three different independent assays. Standard error bars are shown. *b-d*, same as in *a*, but, to stop the refolding reaction rhodanese activity was assayed in the presence  
20 of 10 mM trans-1,2-cyclohexanediaminetetraacetate (CDTA) to inhibit GroEL activity or 0.5 mg/ml of casein to saturate the apical domain.

Maximal refolding yield is obtained at molar ratios of  
25 apical domain and rhodanese of larger than one, from which is estimated a dissociation equilibrium constant of  $>1 \times 10^{-7}$  M.

#### Example 4 - Refolding of cyclophilin A

30

The chaperone activity of sht-GroEL 191-345 and sht-GroEL 191-376 was tested using cyclophilin A. Refolding of cyclophilin A was initiated by diluting 8 M urea denatured protein (100  $\mu$ M) into 100 mM potassium

phosphate buffer pH 7.0, 10 mM DTT to a final concentration of 1  $\mu$ M. The final concentration of GroEL and apical domain in refolding buffer was 7  $\mu$ M and 4  $\mu$ M or 1  $\mu$ M, respectively. Refolding temperature was 25°C.

5 After incubation for the times indicated, cyclophilin activity was measured as described (Fischer G et al (1984) Biomed Biochim Acta 43: 1101-1111). Spontaneous refolding of cyclophilin A occurred to a yield of about 30%, and was finished in less than 1 min. Standard error  
10 was 5%.

Figure 3e shows the refolding of 1  $\mu$ M cyclophilin A in the presence of 7  $\mu$ M GroEL monomer (O), 4  $\mu$ M sht-GroEL 191-345 ( $\bullet$ ), 4  $\mu$ M sht-GroEFL 191-376 ( $\square$ ), or 1  $\mu$ M sht-  
15 GroEL 191-376 ( $\square$ ). 100% activity was obtained with native cyclophilin A. Figure 3e demonstrates that 100% refolding of inactivated cyclophilin A can be achieved with sht-GroEL 191-345 or sht-GroE 191-376 and that this is equivalent to that seen with GroEL.

20 Cyclophilin A refolds only at low yield in the absence of chaperone but refolding is facilitated in the presence of GroEL monomer owing to a transient complex formation (see Zahn et al (1996) FEBS Lett 380: 152-156). A similar  
25 rate constant was found for refolding of cyclophilin A in the presence of intact GroEL monomer and in the presence of GroEL fragments, which within a factor of four did not depend on chaperone concentration (Figure 3e). Maximal  
30 refolding yield was obtained at stoichiometric concentrations of cyclophilin A and apical domain, indicating the formation of 1:1 complex between chaperone fragment and substrate protein during refolding.

Example 5 - Thermal denaturation of sht-GroEL 191-376 and sht-GroEL 191-345

Thermal denaturation monitored by far ultra violet-  
5 circular dichroism (UV-CD) at 222 nm and was carried out  
on a Jasco J720 spectropolarimeter interfaced with a  
Neslab RTE-100 water bath, using a thermostated cuvette  
(Helma) with 1 mm path length. The temperature was  
increased at a linear rate of 50 deg/h. The protein  
10 concentration was 40  $\mu$ M in 10 mM sodium phosphate buffer  
pH 7.0. Data were fitted to a denaturation curve (Pace,  
C N (1990) Trends Biotechnol 8: 93-98) to determine  $T_m$ ,  
the midpoint temperature of denaturation.

15 Thermal denaturation was also monitored by differential  
scanning calorimetry (DSC) and the measurements were  
performed at a protein concentration of  $88 \pm 5$   $\mu$ M in 10 mM  
sodium phosphate buffer pH 7.0, using a Microcal MC-2D  
instrument at a notional scan rate of 60 deg/h. Sample  
20 preparation and data analysis were performed as described  
previously (Johnson C M and Fersht A R (1995)  
Biochemistry 34: 6795-6804). Both proteins exhibit at  
least 50% reversibility in their thermal unfolding as  
judged from the area of endotherms obtained on rescanning  
25 samples. Higher levels of reversibility were obtained at  
lower concentrations or by stopping scans at temperatures  
closer to the main unfolding transition. The low  
temperature transition observed in sht-GroEL 191-376 was  
completely reversible with scans limited in temperatures  
30 below 50°C.

Figure 4a shows the far UV-CD results and Figure 4b shows  
the DSC results. In each Figure, sht-GroEL 191-376 is  
the upper trace and sht-GroEL 191-345 is the lower trace.

The C-terminal helices were found to be flexible. The apical domain and the fragment truncated at position 345 are reversibly denatured by temperature or urea, and the denaturation is not influenced by the N-terminal histidine-tail. There are two cooperative folding transitions, at 34°C and 67°C. At 45°C, the CD spectrum of the apical domain is identical to that of the truncated domain. The C-terminal  $\alpha$ -helices, therefore, must melt at the lower temperature and separately from the "domain core". The second cooperative transition associated with the extra 31 amino acids in sht-GroEL 191-376 was confirmed by DSC (Figure 4b). The calorimetric data are also essentially consistent with the unfolding of the apical domain as a monomer. At physiological temperature, about 50% of helices H11 and H12 are in an unfolded conformation, and thus flexible.

Example 6 - Crystallisation of GroEL fragments and diffraction study

Crystals were obtained from hanging drops initially containing sht-GroEL 191-345 at 23 mg ml<sup>-1</sup>, 11% PEG 4000, 50 mM Tris-HCl pH 8.5 and 100 mM LiSO<sub>4</sub>, equilibrated against reservoirs consisting of 22% PEG 4000, 100 mM Tris-HCl pH 8.5 and 200 mM LiSO<sub>4</sub>. X-ray data were collected from a capillary-mounted crystal at 4°C using a 30 cm Mar Research image plate detector at station 9.6 of the Synchrotron Radiation Source (SRS) at Daresbury, UK ( $\lambda=0.87$  Å). Unless stated otherwise, all data processing, data reduction, electron density syntheses and structural analyses were carried out using CCP4 software (Daresbury Laboratory, Warrington, UK). Indexing and intensity measurements of diffraction data



were carried out with the MOSFLM program suite Leslie A G W in Joint CCP4 and ESF-EACMB Newsletter on Protein Crystallography No 26 (Daresbury Laboratory, Warrington, UK, 1992).

5

The structure was solved by conventional molecular replacement methods, using the program AMORE (Navaza J (1994) Acta Crystallogr A 50: 157-163), and a search model consisting of residues 191-345 of the refined structure of GroEL (Braig et al (1995) Nature Struct Biol 2: 1083-1094). The asymmetric unit contains one protein molecule, corresponding to a solvent content of 51%.

Model rebuilding and refinement was carried out with O (Jones et al (1991) Acta Crystallogr A 47: 110-119), and the structure was refined using X-PLOR (Brünger A T X-PLOR, Version 3.1, A System for Crystallography and NMR (Yale Univ Press, New Haven, CT, 1992)), using Engh and Huber parameters (Engh R A & Huber R (1991) Acta Crystallogr A 47: 392-400). The current model contains 8 water molecules and is complete with the following exceptions, which could not be modelled due to poor or non-existent electron density: residues 302-307 and residues 337-345 from the C-terminus. Electron density for the N-terminal His-tag is also not observed. No residues have disallowed backbone  $\phi\psi$  angles. Table 1 provides a summary of the crystallographic data.

30

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TABLE 1 Summary of crystallographic data

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*Data collection statistics*

Unit cell dimensions	$a = b = 91.67 \text{ \AA}$ , $c = 38.33 \text{ \AA}$
Space group	$P3_121$
Resolution ( $\text{\AA}$ )	22.0-2.5
Measured reflections	21,762
Unique reflections	6,564
Completeness of data (%) <sup>*</sup>	99.4 (96.7)
$R_{\text{merge}}$ (%) <sup>*†</sup>	9.9 (45.1)
$\langle F/\sigma F \rangle$ <sup>*</sup>	19.8 (4.6)
Multiplicity <sup>*</sup>	3.3 (3.0)
<i>Refinement statistics</i>	
Resolution ( $\text{\AA}$ )	8.0-2.5
R-factor/free R-factor (%), $F > 0^\dagger$	21.4/29.1
r.m.s.d bond length ( $\text{\AA}$ )	0.006
r.m.s.d bond angle (deg)	1.42

\* values given in parenthesis are for the highest resolution shell.

† agreement between intensities of repeated measurements

5 of the same reflections and can be defined as:  $\Sigma(I_{h,i} - \langle I_h \rangle) / \Sigma I_{h,i}$ , where  $I_{h,i}$  are individual values and  $\langle I_h \rangle$  is the mean value of the intensity of reflection  $h$

† The free R-factor was calculated with the 10% data omitted from the refinement (test set, prepared using  
 10 DATAMAN (Kleywegt, G.J. & Jones, t.A. Acta. crystallogr. D 50, 178-185 (1994))

Example 7 - The three dimensional structure of sht-GroEL  
191-345

Figure 5a shows the secondary structure representation drawn with MolScript (Kraulis P J (1991) J Appl Crystallogr 24: 946-950) and Raster3D (Meritt E A and Murphy M E P (1994) Acta Crystallogr D 50: 869-873).

5 Helices are labelled as in Braig et al (Braig et al (1995) Nature Struct Biol 2: 1083-1094). N and C refer to the N-terminus (residue 191) and C-terminus (residue 336) of the model.

10 Figure 5b is a backbone representation drawn with programm O (Jones et al (1991) Acta Crystallogr A 47: 110-119), in same orientations as Figure 5a colour-coded according to B-factor of main-chain atoms: blue ( $20 \text{ \AA}^2$ ) to red ( $60 \text{ \AA}^2$ ).

15

Figure 5c shows a representative region of electron density, calculated using refined co-ordinates, viewed along the helices H8 and H9.

20 Crystals of sht-GroEL 191-345 grow in the trigonal space group  $P3_121$  with one molecule per asymmetric unit, giving a solvent content of 51%. The three dimensional structure of the "domain core" was solved by molecular replacement and refined to an R-factor of 21.4% and a  
25 free R-factor of 29.1% for all data between 8.0 and 2.5 Å (Table 1). The quality of the electron density map is shown in Figure 5c.

Overall, sht-GroEL 191-345 has the same fold as the  
30 corresponding region of the intact GroEL protein (Figure 5a): two orthogonal  $\beta$ -sheets forming a  $\beta$ -sandwich, flanked by three  $\alpha$ -helices. The structure is more ordered and better resolved than is the apical domain of the intact protein (Figure 5b): the average B-factor is

42 Å<sup>2</sup>, compared with 97 Å<sup>2</sup> for residues 191-336 of the GroEL structure. Unfortunately, such unusually high disorder in the GroEL structure complicates the interpretation of a structural comparison, in the same way as it can be misleading to use an average of NMR structures for comparison with a crystal structure, and so we have not done so here. In essence, the structure can be described as a well ordered β-sandwich scaffold, flanked by relatively flexible helical and loop regions.

In particular, the B-factors of most of the β-strand, α-helix, and loop structure is about 20, 40, and 60 Å<sup>2</sup>, respectively (Figure 5b). There are two regions of considerable disorder. First, electron density is not found for the C-terminal residues 337-345, which correspond to the first half of α-helix H11. This is an agreement with the results from folding experiments, described above. Second, electron density for residues 302-307 is very poor and fragmented. This region is part of the most disordered segment in the structure of intact GroEL (Braig et al (1995) Nature Struct Biol 2: 1083-1094). The content of α-helix and β-sheet secondary structure of the GroEL fragment (Figure 1) is 48% and 74% higher, respectively, compared with the corresponding region of intact GroEL. There are four additional segments of secondary structure in the new structure (Figures 1 and 5a): residues 299-301 form a short β-strand; residues 201-205, 229-232 and 308-317 form 3<sub>10</sub>-helices.

Example 8 - Refolding chromatography of IGPS (49-252) (indoleglycerol phosphate synthase lacking residues 1-48)

The fragments GroEL(191-345) or GroEL(191-376) were expressed in *E. coli* with a 17-residue N-terminal tail containing 6 histidine residues so that they could be purified using Ni-NTA resin - the  $\text{Ni}^{2+}$  ion which is chelated by the agarose binds to the histidine tag. Initially, the same resin was used to immobilise purified GroEL(191-345) or GroEL(191-376) by their histidine tags for preparative purposes. Subsequently, the purified fragments were also linked covalently to agarose via CNBr activation (Axen R et al (1967) Nature 1302-1304). The immobilised GroEL fragments were found to facilitate the refolding of cyclophilin A with high efficiency.

The apical domain of GroEL (GroEL(191-376)) and the "core" of the apical domain, GroEL(191-345), were cloned and expressed in *E. coli* as fusion proteins containing a 17-residue N-terminal histidine-tail. The fragments were immobilised by two methods.

A). *Immobilised apical domain attached to Ni-NTA resin.* The Ni-NTA resin (from QIAGEN) is a chelating adsorbant composed of a high surface concentration of nitrilo-triacetic acid (NTA) ligand attached to Sepharose CL-6B. The NTA occupies four of six ligand binding sites in the coordination sphere of the  $\text{Ni}^{2+}$  ion, leaving two sites free to interact with the six histidines in the N-terminal tail. Proteins containing one or more 6 x His affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin with an affinity ( $K_d = 10^{-13}$  M, pH 7.8). The stability of the 6 x His/Ni-NTA interaction is unaffected by strong denaturants such as 6M guanidine hydrochloride or 8M urea, or the presence of low levels of  $\beta$ -mercaptoethanol (1-10 mM). 3.5 mL of Ni-NTA resin were equilibrated with

0.1M potassium phosphate at pH 7.8, containing 5 mM  $\beta$ -mercaptoethanol. The GroEL domain was added to saturation of the affinity gel (21 mg of protein per 3.5 mL of gel) and incubated at room temperature for 30 min  
5 with gentle mixing. The gel was packed in a column suitable for FPLC (5 x 100 mm, from Pharmacia) and thoroughly washed with the initial buffer.

*B). Immobilised apical domain attached to CNBr-activated  
10 Sepharose 4B*

To minimise the steric effects and preserve the structure of the binding site in the apical domain, the binding capacity of the gel was reduced by controlled hydrolysis of the activated gel before coupling. 300 mg of freeze-  
15 dried powder were suspended in 50 mM of  $\text{NaHCO}_3$  pH 8.3, washed with the same buffer and re-swollen on a sintered glass filter (G3), then suspended in the buffer and mixed in an end-over-end shaker for 4 h at room temperature. The apical domain, dissolved in the  
20 coupling buffer (0.1M  $\text{NaHCO}_3$ , pH 8.3 and 0.5 M NaCl), was added to the gel suspension (10 mg protein/mL gel) and mixed in an end-over-end shaker for 6 h at room temperature. It was then washed with the coupling buffer. The remaining active groups were blocked by  
25 adding 2.5 M ethanolamine pH 8 and shaking for 4 h at room temperature. Uncoupled apical domain was removed by washing with five cycles of alternately high and low pH buffer solution (Tris-HCl 0.1M pH 7.8 containing 0.5M NaCl followed by acetate buffer (0.1M, pH 4 plus 0.5M  
30 NaCl). The gel was finally washed with 0.1 M potassium phosphate at pH 7.8, containing 5 mM 2-mercaptoethanol (refolding buffer).

The immobilised fragments were used in a chromatography column (Figure 6). Denatured protein in urea was added to the column and developed with a refolding buffer. The protein eluted as from a conventional binding column, its passage was retarded and the peak could be characterised by a retention time. Some mutants of indoleglycerol phosphate synthase (IGPS) were expressed *E. coli* and isolated as inclusion bodies. These reprecipitate on attempts to renature them after dissolving at high concentrations in urea, and attempts at low protein concentrations yielded soluble material of non-native conformation. The mutant IGPS (49-252) (1 x 22 kDa), which lacks the first 48 amino acid residues, was obtained on chromatography on the GroEL(191-345) column in a 92% yield of material that had 100% binding activity (Figure 6). The term "refolding chromatography" can be used to describe the phenomenon of refolding by passage through the column.

In more detail, a column of ht-GroEL 191-345 immobilised on Ni-NTA agarose (3.0 mL) was loaded with 2 nmol of IGPS (49-252) dissolved in 20 µL of 8 M urea, and the column was developed with refolding buffer (0.1 M potassium phosphate at pH 7.8, containing 5 mM 2-mercaptoethanol) using a Waters 625 LC HPLC system. In Figure 6, the two peaks are seen which are described by:  $K_{av} = (V_e - V_o)/(V_t - V_o)$ , where  $V_t$  is the total volume of gel in the column,  $V_o$ , the void volume, and  $V_e$  the volume at the maximum of the peak. A value of  $K_{av}$  indicates that the protein interacts with the support. Peak 1 had  $K_{av} = 1.0$  (9.6 % of the total area) and peak 2  $K_{av} = 1.7$  (90.4%). The protein in peak 2 was recovered in 1.2 mL, and contained 1.85 nmol of truncated IGPS (92.5 % yield).

It displayed a circular dichroism spectrum characteristic of a native a/b protein and bound  $^3\text{H}$ -rCdRP (tritium-labelled reduced 1[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate), a specific inhibitor of IGPS, with a stoichiometry of 1.0. Initially denatured cyclophilin chromatographed with two peaks, an inactive at  $K_{av} = 0.38$  and an active at  $K_{av} 1.43$

Example 9 - Batchwise renaturation of cyclophilin A on Ni-NTA agarose gels

This experiment uses batchwise mixing of materials. A solution of denatured cyclophilin in 8 M urea was diluted 100 fold with Ni-NTA-immobilised GroEL(191-345) in a refolding buffer (see Table II below). After gentle mixing for 30 min, the cyclophilin was recovered in 84% yield of protein. Control experiments (Table II) in which the protein was denatured in 8 M urea and added to the refolding buffer alone, or mixed with agarose that had not been linked to GroEL fragments, gave cyclophilin that had only 20% of the activity of the starting material before denaturation. The cyclophilin used for the denaturation experiments had 88% of the activity of the purest samples previously obtained by the inventors. The specific activity of the material obtained from the GroEL(191-345)-agarose resin (covalently linked) was 126% of that of the previous purest samples. Further, the total recovery of activity was 25% more than that initially present. Thus, the immobilised GroEL fragment had "reconditioned" the cyclophilin A by converting inactive material into active.

In more detail, suspension of 200  $\mu\text{L}$  of gel (wet, sedimented volume) was mixed with refolding buffer (100



mM phosphate buffer, pH 7.8 plus 5 mM 2-mercaptoethanol) to give a volume of 990  $\mu$ L. 10  $\mu$ L of cyclophilin (from a 100  $\mu$ M stock solution in refolding buffer + 8M urea, = 1 nmol of cyclophilin A) were added and the suspension was  
5 mixed in an up-down mixer for 30 min at room temperature.

The gel suspension was centrifuged to separate the supernatant (~800  $\mu$ L). The gel pellet was washed in miniprep columns and the eluate added to the supernatant  
10 to give about 900  $\mu$ L. The protein concentration was determined from the  $A_{280}$  nm. Cyclophilin activity was measured in the supernatant as described in (Makino Y et al (1993) FEBS Lett 336: 363-367). The sample prior to  
15 denaturation had 88% of the specific activity of the highest activity of native cyclophilin previously obtained by the inventors. The control is agarose alone, without Ni. The results are shown in table II below.

Table II

	Gel type	Protein yield	Cyclophilin		Cyclophilin specific activity relative to "pure" native
			activity yield	(%)	
5		(%)	(%)	(%)	
10					62
	GroEL 191-345 agarose	84	98	103	
	GroEL 191-376 agarose	81	105	115	
	GroEL 191-345 agarose	87	125	126	
	Agarose (control) <sup>5</sup>	72	17	20	
15	Native cyclophilin (control)			88	

The procedure was applied to other proteins used in the laboratory. Glucosamine 6-phosphate deaminase (6 x 30 kDa) (Oliva G et al (1995) Structure 3: 1323-1332) normally regains only 10 % or less activity after  
5 renaturation from urea denaturation. A 100 % yield was obtained on batchwise treatment with GroEL(191-345)-agarose. Further, a sample that had lost all activity on storage in solution in 50% glycerol/water at -20 °C for 5 years also regained 100 % activity with this treatment  
10 after dissolving in urea.

Example 10 - Residues of GroEL implicated in binding peptides

15 The X-ray crystal structure of GroEL 191-376 with the 17 residue N-terminal tail shows that seven residues of the tail of one molecule bind in the active site of the other. Residues 230-271 are in the binding site. All residues are shown in Figure 8 in which large, bold and  
20 underlined residues are those detected by X-ray crystal structure of ht GroEL 191-376 as being involved in protein binding.

The X-ray crystal structure shows that 193-336 should be  
25 reasonably stable. The 193-337 fragment was cloned and expressed and found to be stable. Therefore, residues 191 and 192 may be omitted.

Figure 8

---

	190	VEGMOFDRGY	LSPYFINKPE	TGAVELESPF	ILLADKKISN
	230	<u>I</u> REMLPVLEA	VAKAGKPLLI	IAEDVEGEAL	<u>ATAVVNTIRG</u>
5	270	<u>I</u> VKVAVKAP	GFGDRRKAML	QDIATLTGGT	WISEEIGMEL
	310	EKATLEDLGQ	AKRVVINKDT	TTIIDGVGEE	AAIQGRVAQI

---

10 Example 11 - Minimal size of functional unit - refolding activity of polypeptides

Polypeptide fragments of *E. coli* GroEL were synthesised on a protein synthesiser according to methods and using equipment well known to a person skilled in the art.

15 Longer fragments were generated by synthesising smaller fragments and then linking them together. The fragments were then tested by their ability to refold rhodanese, cyclophilin A, glucosamine-6-phosphate deaminase and IGPS 49-252 as described above.

20

The following fragments were made and tested:

a) 245-256, 244-257 ...et seq... 192-309, 191-310  
[55 separate fragments].

25

b) 235-301, 234-302 ...et seq... 193-343, 192-344  
[44 separate fragments].

30 Refolding activity above background controls was found for all polypeptides comprising at least amino acid residues 230-271 of GroEL.

Example 12 - Alignment of consensus binding sequences of  
cpn60 family members

5 Figure 9 shows sequences in OWL database release 28.1  
containing clear homology to apical domain of GroEL  
(residues 191-375) in PDB structure pdblgrl.ent. OWL is  
a non redundant database merging SWISS-PROT, PIR (1-3),  
GenBank (translation) and NRL-3D. Consensus sequence =  
10 residues 230-271 inclusive) containing peptide-binding  
site (as identified by crystal structure analysis and  
polypeptide binding studies). X = residue in peptide-  
binding site in the X-ray crystal structure of mini  
chaperone. The GroEL *E. coli* chaperone sequence is shown  
in italics.

15

*n:349spec*

## Claims:

1. A chaperone polypeptide having an amino acid sequence selected from at least amino acid residues 230-271 but no more than residues 150-455 or 151-456 of a GroEL sequence substantially as shown in Figure 7, or a corresponding sequence of a substantially homologous chaperone polypeptide, or a modified, mutated or variant thereof having chaperone activity.
2. Monomeric polypeptide having chaperone activity and incapable of multimerisation in solution.
3. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

1 X X X 2 X X 3 4 X X 5 X X X X X X X X X X X X X X X X 6 X  
 X 7 8 X 9 10 11 X X 12 X 13 14

- wherein 1 is selected from amino acid residues:  
 I, M, L, V, S, F or A;  
 wherein 2 is selected from: L, I, P, V or A;  
 wherein 3 is selected from: L, E, V, H or I;  
 wherein 4 is selected from: E, A, R, L, Q, or N;  
 wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;  
 wherein 6 is selected from: E, D or G;  
 wherein 7 is selected from: A, P, S, T, G or L;  
 wherein 8 is selected from: T, A, N, S or V;  
 wherein 9 is selected from: V, L, I or A;  
 wherein 10 is selected from: V, L, I, F or H;  
 wherein 11 is selected from: N, S or L;  
 wherein 12 is selected from: R, K, N, Q, L or S;  
 wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
 wherein 14 is selected from: V, I, L, F, D or T; and

wherein the X's represent a peptide bond or bonds or at least one amino acid residue,

5 or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

4. A chaperone polypeptide which, when in solution, remains  
10 monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including at least one protein binding active site motif moiety selected from:

(a) 1 X X X 2 X X 3 4 X and

15

(b) X 5 X X X X X X X X X X X X X 6 X X 7 8 X 9 10  
11 X X 12 X 13 14

wherein 1 is selected from amino acid residues:

20

I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

25

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;

wherein 10 is selected from: V, L, I, F or H;

30

wherein 11 is selected from: N, S or L;

wherein 12 is selected from: R, K, N, Q, L or S;

wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;

wherein 14 is selected from: V, I, L, F, D or T; and

35

wherein X is at least one amino acid residue,

or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

- 5 5. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

10 I X X X L X X L E X X A X X X X X X X X X X X X X X X E X  
X A T X V V N X X R X I V

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino  
15 acid residues has undergone a conservative substitution.

6. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including at least one  
20 protein binding active site motif moiety selected from:

(a) I X X X L X X L E X

(b) X A X X X X X X X X X X X X X X X E X X A T X V V  
25 N X X R X I V

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.  
30

7. A chaperone polypeptide as claimed in any one of claims 3 to 6 in which the active site motif or an active site motif moiety includes the conserved sequence:

35 PLL(V) I(V) IA(S) EDV(I) EGEAL



in which amino acid symbols in parenthesis are alternatives to the immediately preceding symbol reading left to right.

8. Monomeric polypeptide having chaperone activity and  
5 incapable of multimerisation characterised in that in the  
absence of ATP the polypeptide has a protein refolding  
activity of more than 50%, preferably 60%, even more  
preferably 75%, said refolding activity being determined by  
10 contacting the polypeptide with an inactivated protein of  
known specific activity prior to inactivation, and then  
determining the specific activity of the said protein after  
contact with the polypeptide, the % refolding activity being:

15 
$$\frac{\text{specific activity of protein after contact with polypeptide}}{\text{specific activity of protein prior to inactivation}} \times \frac{100}{1}$$

9. A polypeptide as claimed in any preceding claim, wherein  
the chaperone activity is determined by the refolding of  
cyclophilin A.

20

10. A polypeptide as claimed in claim 9 wherein 8M urea  
denatured cyclophilin A (100 $\mu$ M) is diluted into 100mM  
potassium phosphate buffer pH7.0, 10mM DTT to a final  
concentration of 1 $\mu$ M and then contacted with at least 1 $\mu$ M of  
25 said polypeptide at 25°C for at least 5 min, the resultant  
cyclophilin A activity being assayed by the method of Fischer  
G et al (1984) Biomed Biochim Acta 43: 1101-1111.

11. A polypeptide as claimed in any preceding claim being an  
30 hsp60 polypeptide, preferably a GroEL polypeptide.

12. A polypeptide as claimed in any preceding claim which  
comprises at least an amino acid sequence selected from GroEL  
residues:

35

- 5 (a) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334,  
191-335, 191-336, 191-337, 191-338, 191-339, 191-340,  
191-341, 191-342, 191-343, 191-344, 191-345, 191-346,  
191-347, 191-348, 191-349, 191-350, 191-351, 191-352,  
191-353, 191-354, 191-355, 191-356, 191-357, 191-358,  
191-359, 191-360, 191-361, 191-362, 191-363, 191-364,  
191-365, 191-366, 191-367, 191-368, 191-369, 191-370,  
191-371, 191-372, 191-373, 191-374, 191-375 or 191-376,  
or
- 10 (b) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334,  
192-335, 192-336, 192-337, 192-338, 192-339, 192-340,  
192-341, 192-342, 192-343, 192-344, 192-345, 192-346,  
192-347, 192-348, 192-349, 192-350, 192-351, 192-352,  
15 192-353, 192-354, 192-355, 192-356, 192-357, 192-358,  
192-359, 192-360, 192-361, 192-362, 192-363, 192-364,  
192-365, 192-366, 192-367, 192-368, 192-369, 192-370,  
192-371, 192-372, 192-373, 192-374, 192-375 or 192-376,  
or
- 20 (c) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334,  
193-335, 193-336, 193-337, 193-338, 193-339, 193-340,  
193-341, 193-342, 193-343, 193-344, 193-345, 193-346,  
193-347, 193-348, 193-349, 193-350, 193-351, 193-352,  
25 193-353, 193-354, 193-355, 193-356, 193-357, 193-358,  
193-359, 193-360, 193-361, 193-362, 193-363, 193-364,  
193-365, 193-366, 193-367, 193-368, 193-369, 193-370,  
193-371, 193-372, 193-373, 193-374, 193-375 or 193-376,  
or
- 30 (d) 230-271, 229-271, 229-272, 228-272, 228-273, ...et  
seq... 194-328, 194-329, or

the equivalent residues of substantially homologous  
35 chaperonins, or a modified, mutated or variant sequence  
thereof.

13. A polypeptide as claimed in claim 8, wherein the selected amino acid sequence is selected from 230-271, 191-345, 191-376, or 193-337 of GroEL, or the equivalent residues  
5 of substantially homologous chaperonins, or a modified, mutated or variant sequence thereof.

14. A polypeptide as claimed in any preceding claim further comprising a polyamino acid sequence, preferably an N-  
10 terminal polyamino acid sequence.

15. A polypeptide as claim in claim 14, wherein the polyamino acid sequence is a polyhistidine sequence.

15 16. A polypeptide as claimed in claim 14 or claim 15, wherein the polyamino acid sequence includes a cleavage site cleavable by a cleavage agent, preferably said cleavage agent is thrombin.

20 17. A polypeptide as claimed in any one of claims 14 to 16 wherein the further polyamino acid sequence comprises a number of amino acid residues in the range 2 to 500, preferably 5 to 100, more preferably 17 to 39.

25 18. A polypeptide as claimed in any preceding claim and is immobilised form, optionally immobilised to a chromatographic matrix, preferably an agarose resin.

19. A polypeptide as claimed in claim 18, wherein the  
30 agarose resin is a nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin.

20. A polypeptide as claimed in any preceding claim fused to a heterologous protein or polypeptide.

21. A recombinant polypeptide as claimed in any preceding claim.

22. An isolated nucleic acid molecule comprising a  
5 nucleotide sequence encoding a polypeptide as defined in any one of claims 1 to 21 or a nucleotide sequence hybridisable thereto and optionally encoding a polypeptide having chaperone activity.

10 23. A recombinant nucleic acid molecule for use in cloning and/or expressing a nucleic acid sequence, said recombinant nucleic acid molecule comprising:

15 (a) a nucleotide sequence encoding amino acid residues 191-376 of GroEL, or

(b) a nucleotide sequence encoding amino acid residues 191-345 of GroEL, or

20 (c) a nucleotide sequence encoding amino acid residues 193-337, or

(d) a nucleotide sequence encoding amino acid residues of GroEL selected from amino acid residues:

25

(i) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334,  
191-335, 191-336, 191-337, 191-338, 191-339, 191-340,  
191-341, 191-342, 191-343, 191-344, 191-345, 191-346,  
191-347, 191-348, 191-349, 191-350, 191-351, 191-352,  
30 191-353, 191-354, 191-355, 191-356, 191-357, 191-358,  
191-359, 191-360, 191-361, 191-362, 191-363, 191-364,  
191-365, 191-366, 191-367, 191-368, 191-369, 191-370,  
191-371, 191-372, 191-373, 191-374, 191-375 or 191-376,  
or

35

- (ii) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334,  
192-335, 192-336, 192-337, 192-338, 192-339, 192-340,  
192-341, 192-342, 192-343, 192-344, 192-345, 192-346,  
192-347, 192-348, 192-349, 192-350, 192-351, 192-352,  
5 192-353, 192-354, 192-355, 192-356, 192-357, 192-358,  
192-359, 192-360, 192-361, 192-362, 192-363, 192-364,  
192-365, 192-366, 192-367, 192-368, 192-369, 192-370,  
192-371, 192-372, 192-373, 192-374, 192-375 or 192-376,  
or  
10
- (iii) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334,  
193-335, 193-336, 193-337, 193-338, 193-339, 193-340,  
193-341, 193-342, 193-343, 193-344, 193-345, 193-346,  
193-347, 193-348, 193-349, 193-350, 193-351, 193-352,  
15 193-353, 193-354, 193-355, 193-356, 193-357, 193-358,  
193-359, 193-360, 193-361, 193-362, 193-363, 193-364,  
193-365, 193-366, 193-367, 193-368, 193-369, 193-370,  
193-371, 193-372, 193-373, 193-374, 193-375 or 193-376,  
or  
20
- (e) 230-271, 229-271, 229-272, 228-272, 228-273, ...et  
seq... 194-328, 194-329, or
- (f) a nucleotide sequence hybridisable to any of (a), (b),  
25 (c), (d) or (e) above and encoding a monomeric  
polypeptide having chaperone activity, or
- (g) degenerate nucleotide sequences corresponding to (a),  
(b), (c), (d), (e) or (f) above.  
30
24. A vector comprising a nucleic acid as claimed in claim  
22 or claim 23.
25. A host cell transformed with a nucleic acid as defined  
35 in claim 22 or claim 23 or vector as defined in claim 24.

26. A method of making a polypeptide as defined in any one of claims 1 to 21 comprising transforming a host cell with a nucleic acid encoding said polypeptide, culturing the transformed cell and expressing said polypeptide.

5

27. A method of making a polypeptide as claimed in claim 26 wherein the nucleic acid is as defined in claim 22 or claim 23.

10 28. A method as claimed in claim 26 or claim 27, wherein the expressed polypeptide product is subject to cleavage.

29. A pharmaceutical formulation comprising a polypeptide of any one of claims 1 to 21, optionally together with a  
15 diluent, carrier or excipient.

30. A polypeptide as defined in any one of claims 1 to 21 for use in the treatment of disease.

20 31. The use of a polypeptide as defined in any one claims 1 to 21 in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure.

32. A nucleic acid molecule as defined in claims 22 or claim  
25 23 for use in the treatment of disease.

33. The use of nucleic acid molecule as defined in claim 22 or claim 23 in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide  
30 structure.

34. A method of reconditioning a molecule preferably a protein comprising contacting said protein with a polypeptide of any one of claims 1 to 21.

35

35. A method as claimed in claim 34, wherein the protein is subjected to inactivation or denaturation prior to contacting with said polypeptide.

5 36. A method as claimed in claim 34 or claim 35, wherein the polypeptide is immobilised to a solid phase.

37. A method as claimed in claim 33 or claim 34, wherein the polypeptide is immobilised to a solid phase, preferably a  
10 chromatographic matrix, and the contacting of protein and polypeptide is carried out by applying the protein to the top of a bed of the matrix packed in a column and then eluting the polypeptide through the column.

15 38. Use of a polypeptide as claimed in any one of claims 1 to 21 for altering the structure of a molecule.

39. The use of claim 38, wherein the molecule is a protein or polypeptide and the alteration in structure is by folding  
20 unfolding or refolding.

40. The use of claim 38 or claim 39, wherein the stoichiometry between the polypeptide and the molecule being altered is about 1:1.

25

41. Use of a polypeptide as defined in any one of claims 1 to 21 in the purification or increase in yield, specific activity or quality of biological molecules, preferably said polypeptide being attached to a support.

30

42. A kit for reconditioning or refolding a molecule, preferably a protein, comprising a polypeptide of any one of claims 1 to 21 immobilised to a solid phase and a container for holding said solid phase polypeptide.

35

43. Use of a polypeptide as defined in any one of claims 1 to 21 in the production of a protein or polypeptide by recombinant means, wherein the said polypeptide is co-expressed with the protein or polypeptide thereby to improve the yield or quality of the protein or polypeptide.

44. An antibody reactive against a polypeptide as defined in any one of claims 1 to 21.

45. An antibody as claimed in claim 44 for use in the treatment of disease.

46. The use of an antibody as claimed in claim 44 in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure.

47. A method of treating disease in which an effective amount of a polypeptide of any one of claims 1 to 21 is administered.

48. A method of treating disease which comprises administering an effective amount of an inhibitor of the chaperone activity of a polypeptide of any one of claims 1 to 21.

49. A method as claimed in claim 48 wherein said inhibitor is an antibody.

50. A method of treating disease by gene therapy which utilises a construct encoding a polypeptide of any one of claims 1 to 20 or an antagonist thereof.

n:/349c1



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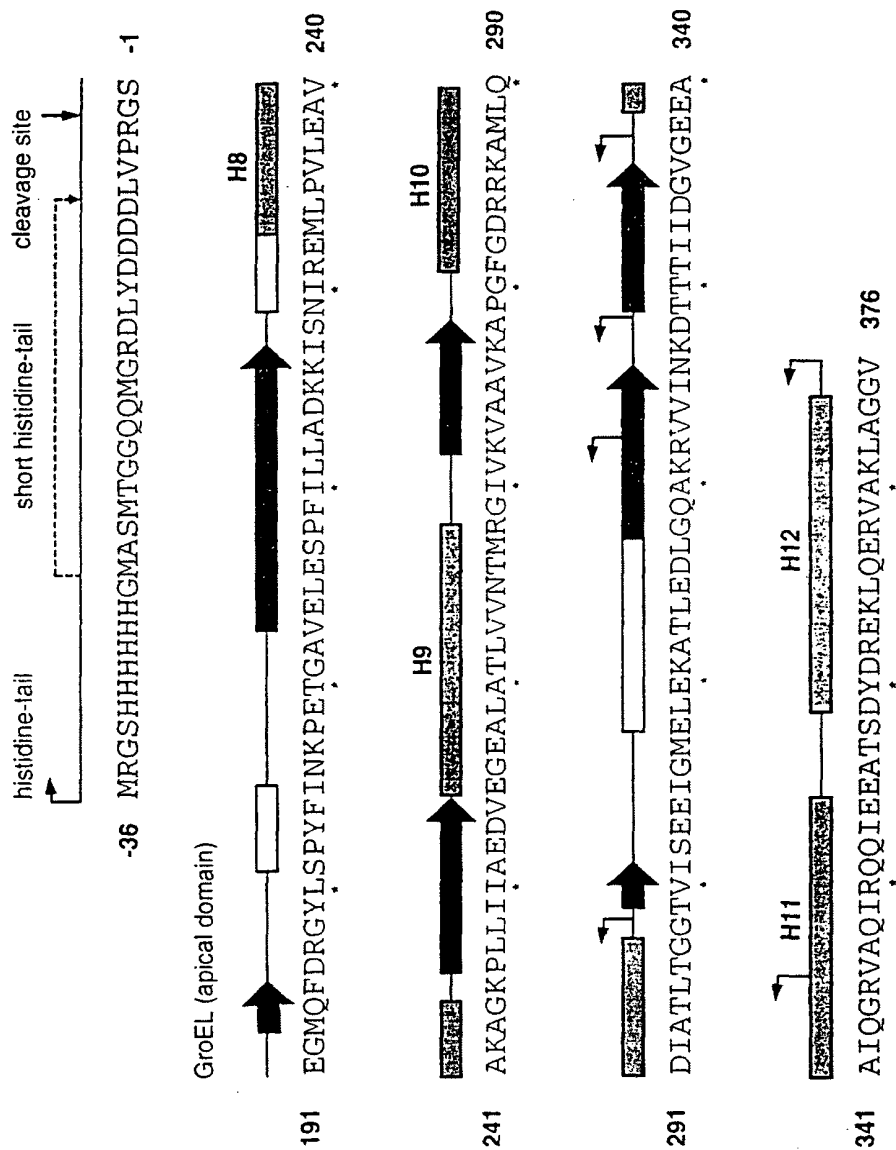


Figure 1

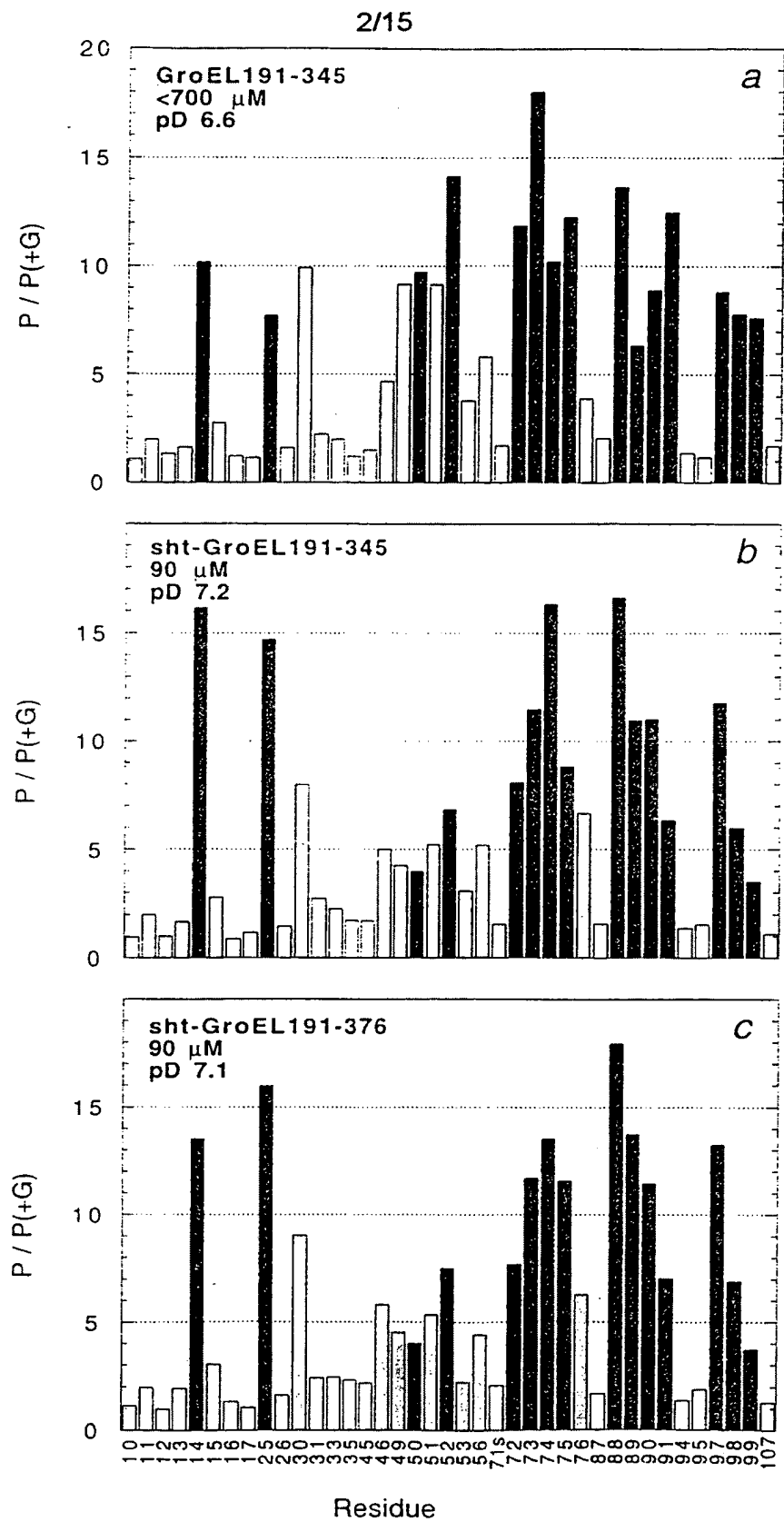


Figure 2a-c

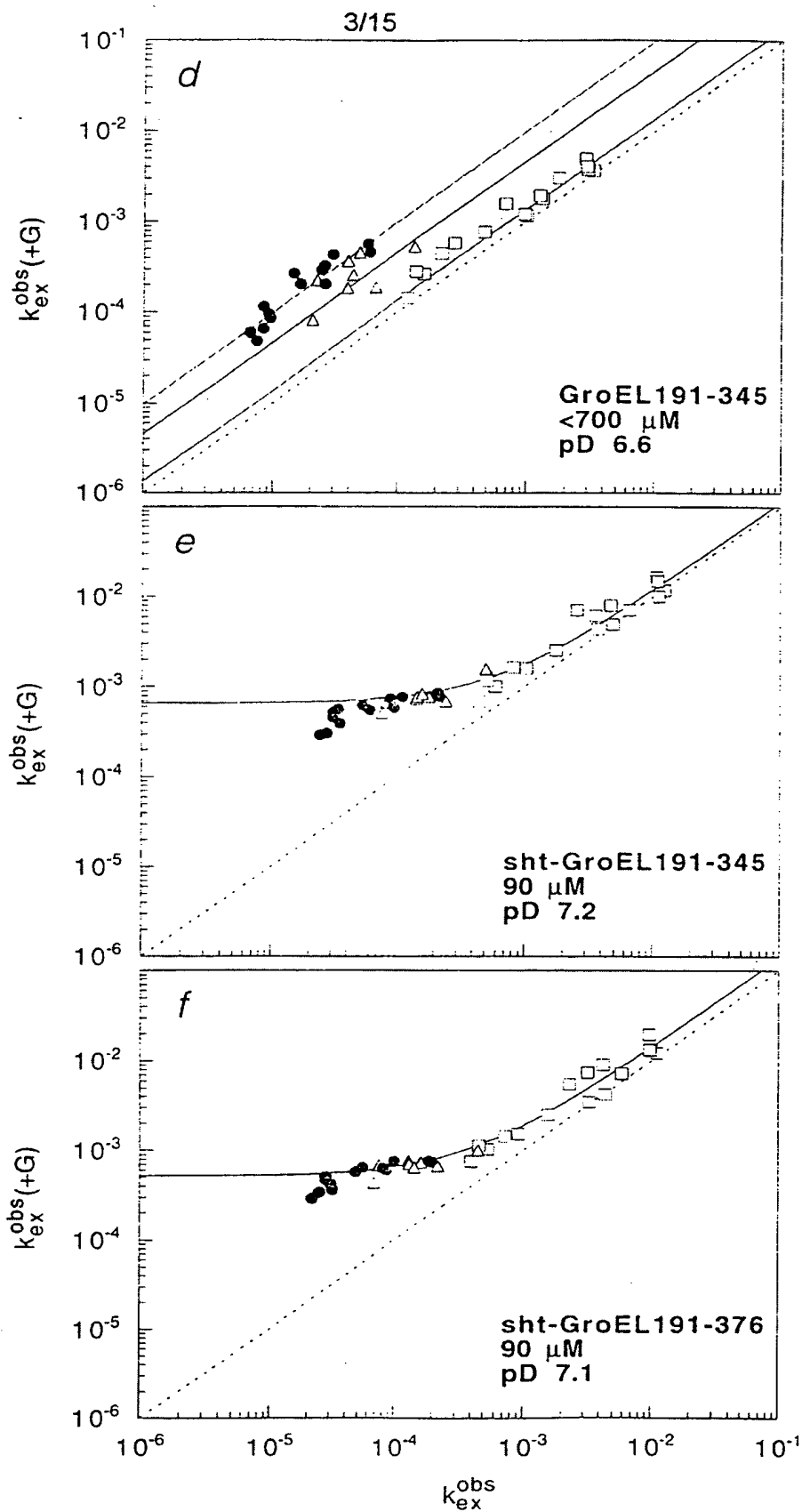


Figure 2d-e

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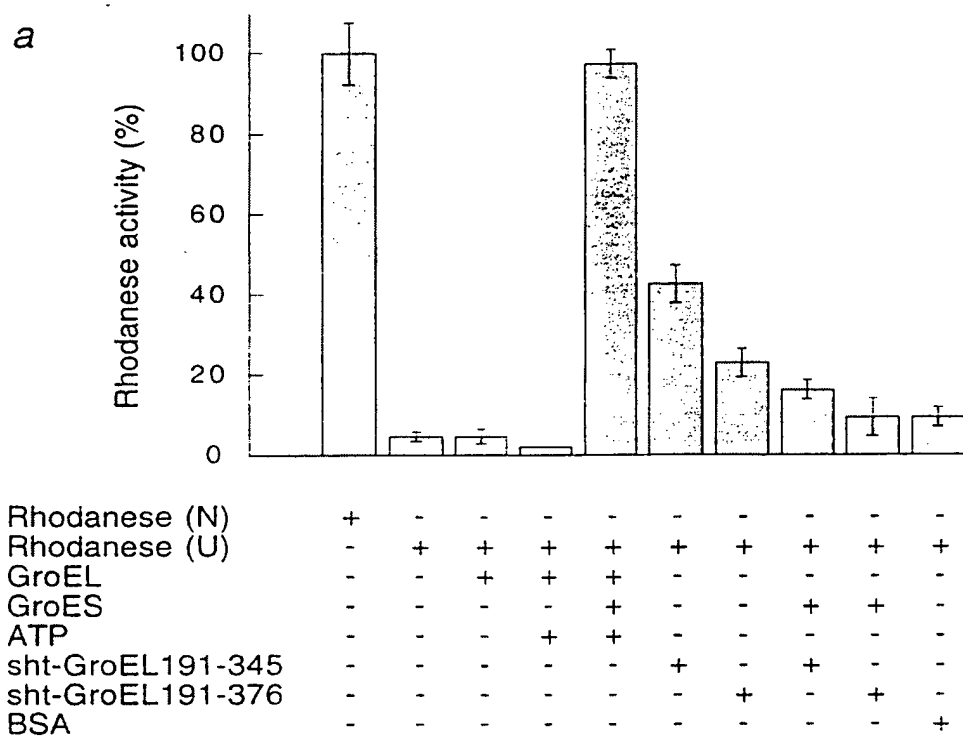


Figure 3a

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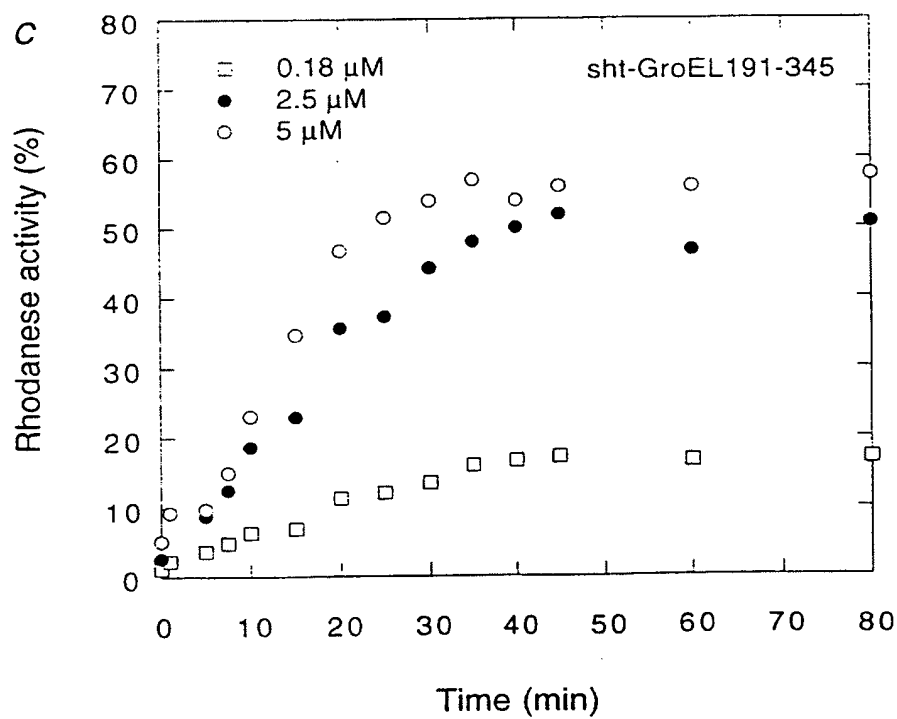
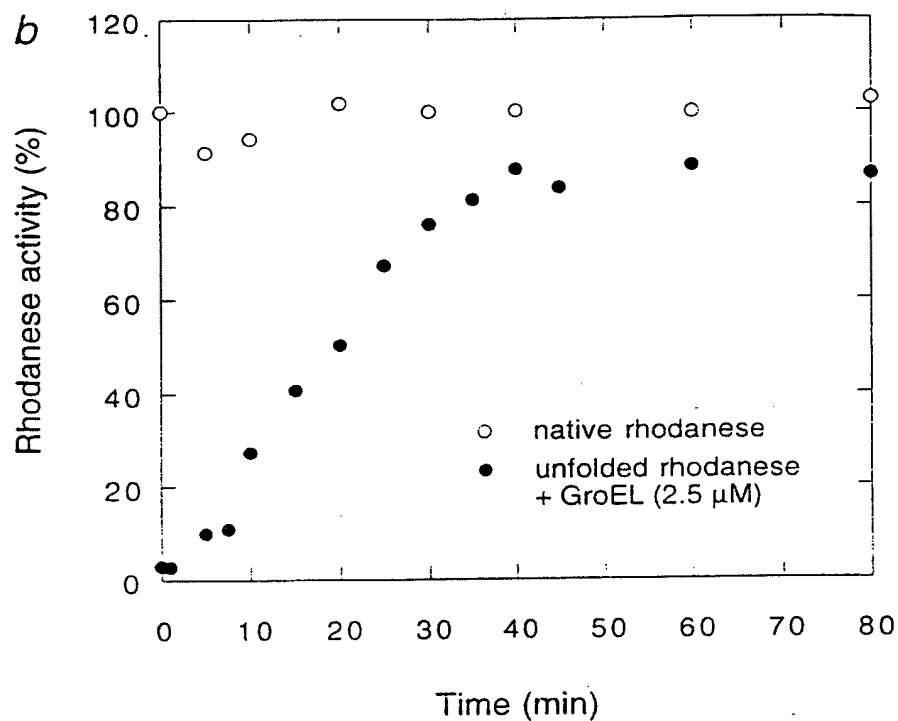


Figure 3b-c

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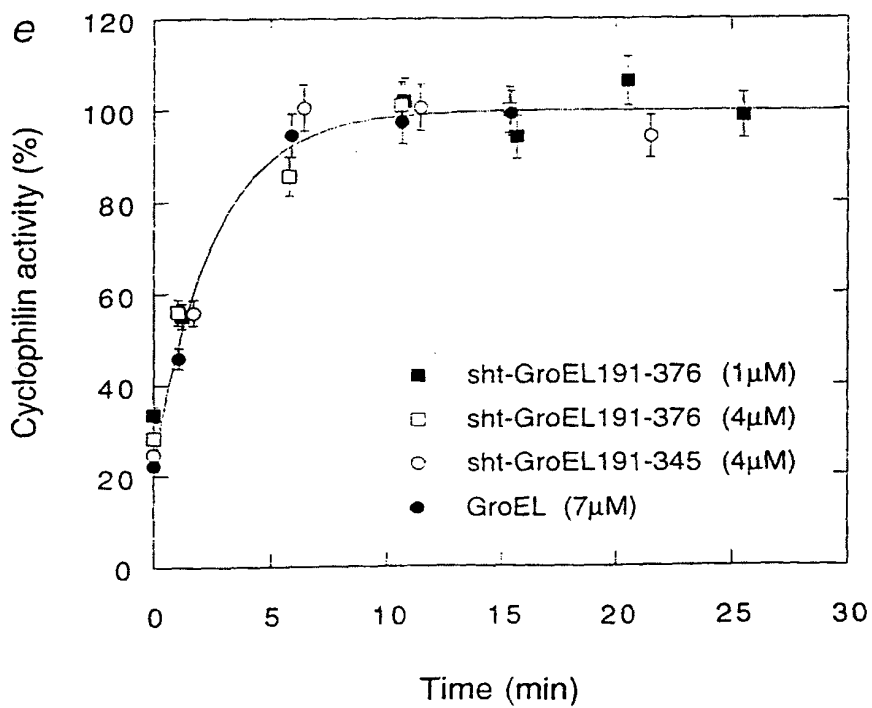
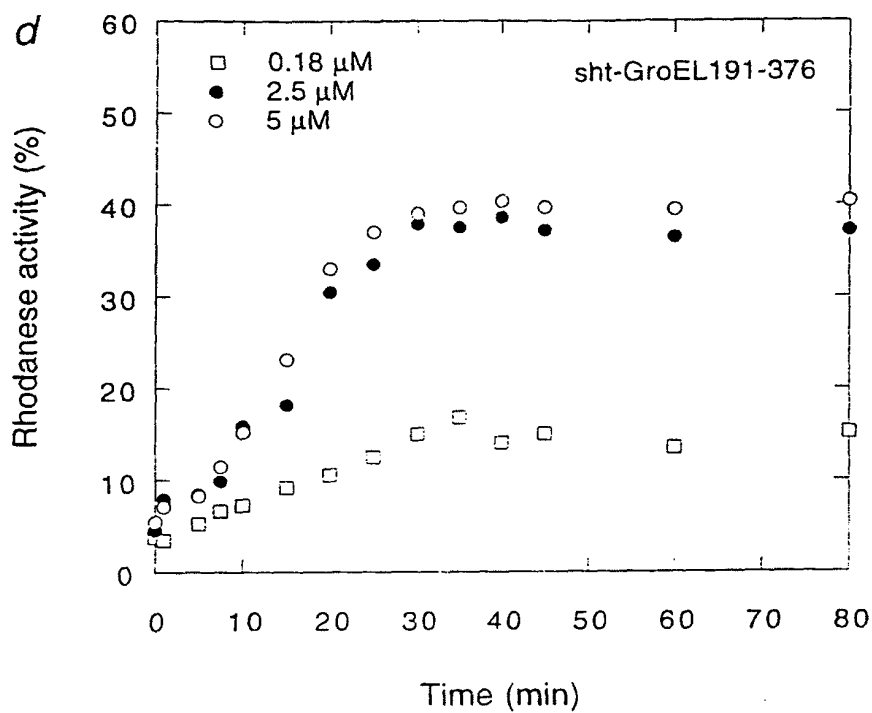


Figure 3d-e

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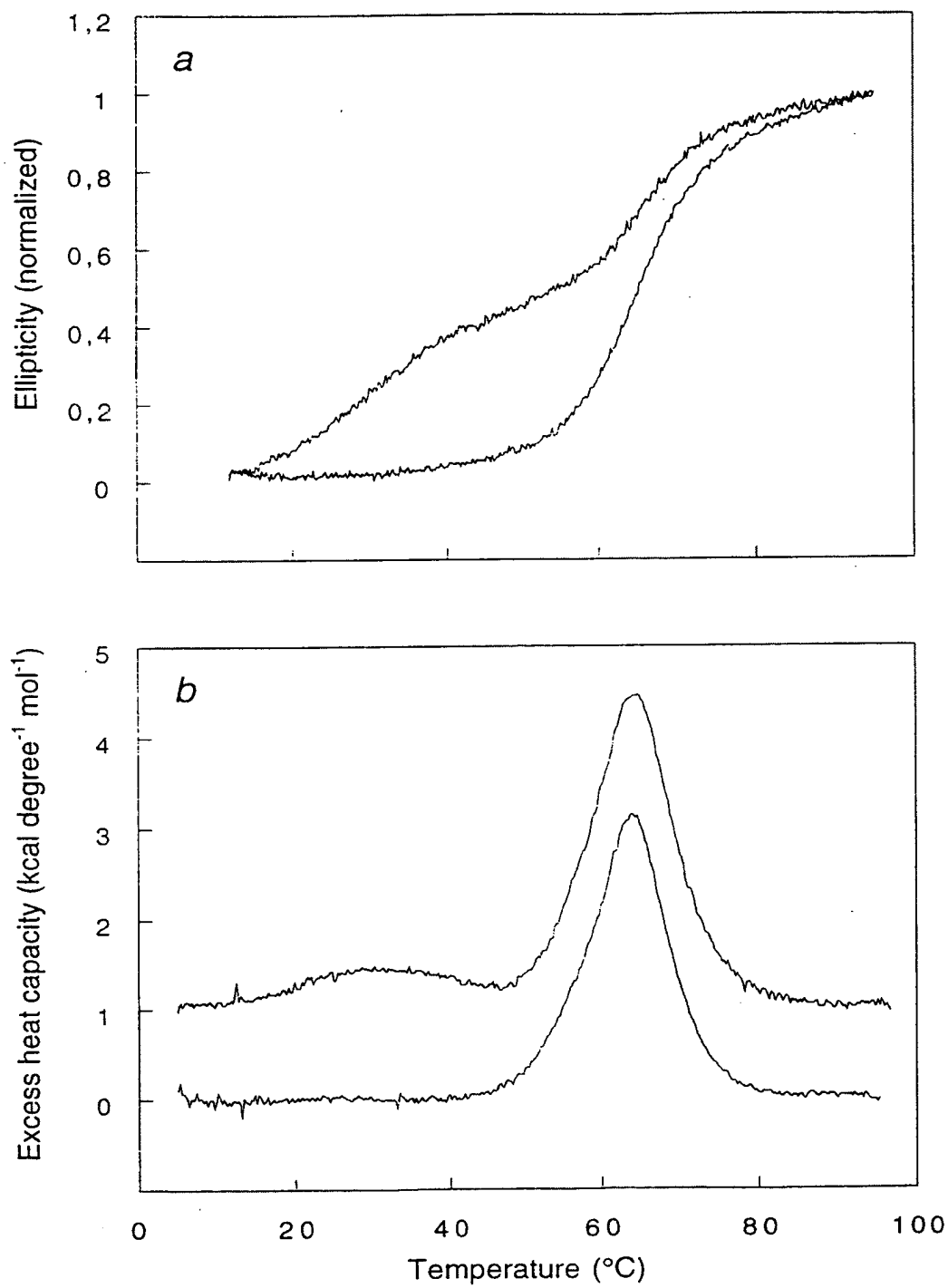


Figure 4

SUBSTITUTE SHEET (RULE 26)

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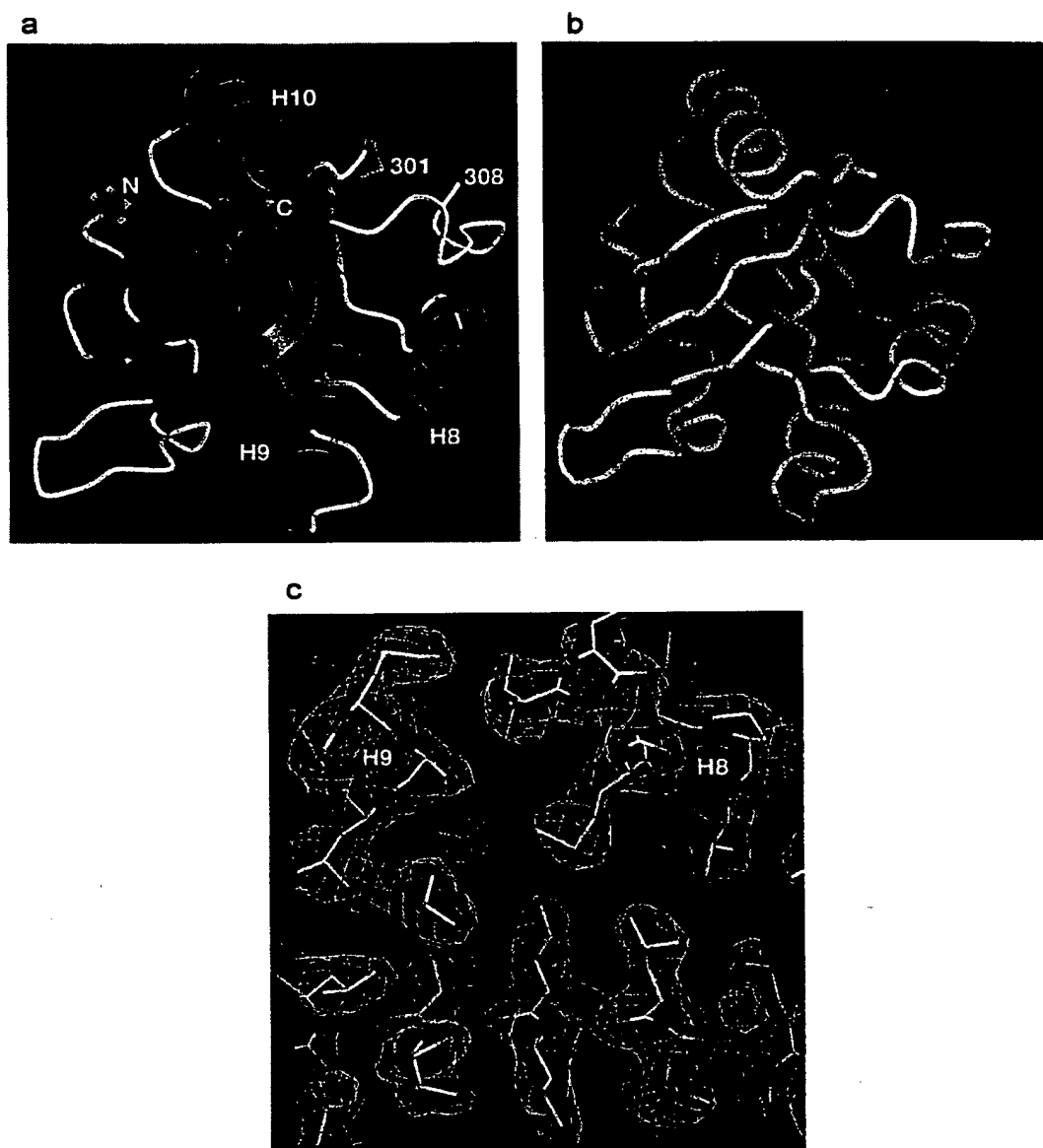


Figure 5



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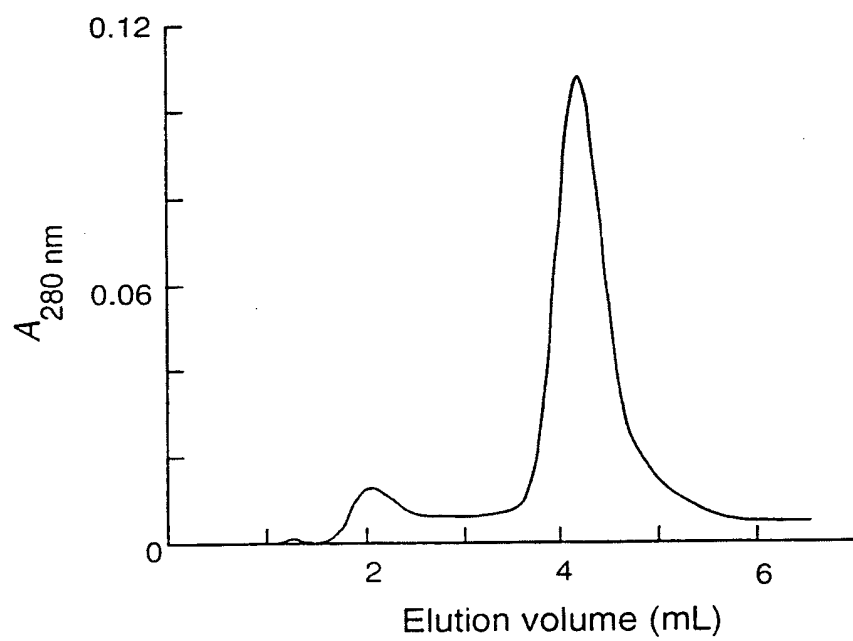


Figure 6

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AAKDVKFGND	ARVKMLRGVN	VLADAVKVTL	GPKGRNVVLD	KSFGAPTITK	DGVSVAREIE	M	1
LEDKFENMGA	QMVKEVASKA	NDAAGDGTTT	ATVLAQAIIT	EGLKAVAAGM	NPMDLKRIGD		61
KAVTAAVEEL	KALSVPCSDS	KALAQVGTIS	ANSDETVGKL	IAEAMDKVGK	EGVITVEDGT		121
GLQDELDVVE	GMQFDRGYLS	PYFINKPETG	AVELESPFIL	LADKKISNIR	EMLPVLEAVA		181
KAGKPLLIIA	EDVEGEALAT	AVVNTIRGIV	KVAAVKAPGF	GDRRKAMLQD	IATLTGGTVI		241
SEEIGMELEK	ATLEDLGQAK	RVVINKDTT	IIDGVGEEAA	IQGRVAQIRQ	QIEEATSDYD		301
REKLQERVAK	LAGGVAVIKV	GAATEVEMKE	KKARVEDALH	ATRAAVEEGV	VAGGGVALIR		361
VASKLADLRG	QNEDQNVGIK	VALRAMEAPL	RQIVLNCGEE	PSVVANTVKG	GDGNYGYNAA		421
TEEYGNMIDM	GILDPTKVTR	SALQYAASVA	GLMITTECMV	TDLPKNDAAD	LGAAGGMGGM		481
GGMGGM							541
							548

Figure 7

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	230	240	250	260	270
	*	*	*	*	*
	x	x	x	xx	xx
	x	x	x	xx	xx
CH60_ECOLI_190	IREMLPVLEAV.				
CH60_SALTI_190	IREMLPVLEAV.				
S56371_191	IREMLPVLEAV.				
CH60_LEPIN_190	MKDLIHILEKV.				
S47530_191	LKEMPLILEQT.				
LPNHTPEG_190	IREMLSVLEGV.				
CH60_ACTAC_189	IRELLPVLEGV.				
JC4519_191	IRELLPVLEGV.				
CH60_BRUAB_191	LQALLPVLEAV.				
CH60_HAEIN_191	IRELLPVLEGV.				
CH60_CAUCR_190	LQPLPVLEAVV.				
CH60_AMOPS_190	IRDMLSVLEAV.				
CH60_HAEDU_191	IREILPVLEAV.				
CH61_RHIME_191	LQAMPLPVLEAV.				
CH60_LEGMI_190	IRDMLSVLEAV.				
CH60_YEREN_191	IREMLPVLEAV.				
CH63_BRAJA_190	LNELLPLLEAV.				
CH60_PORGI_191	LKEMPLILEQT.				
S52901_191	IREMLPVLEAV.				
S26423_191	IREMLPVLEAV.				
RSU373691_191	LQPMVPLEAV.				
CH62_BRAJA_190	LNELLPLLEAV.				
CH60_ACYPS_191	VREMLPILSV.				
CH63_RHIME_191	LQAMPLVLERV.				
YEPHSPCRP1_191	IREMLPVLEAV.				
CH60_BORPE_191	IRDLLPVLEQV.				

Figure 9(a)

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BRUGRO1 189      LQALLPVLEAV.....VQTSKPLIIAEDVEGERLATLVVVKLRGGL
CH60_PSEAE 191    IREMLPVLEAV.....AKAGRPLIIAEDVEGEALATLVVNNMRGIV
CH60_BARBA 190    LQSLLPVLEAV.....AQSGKPLIIAEDVEGEALATLVVVKLRGGL
BAOB63A 191      LQSLLPVLEAV.....VQSGKPLIIAEDVEGEALATLVVVKLRGGL
CH60_BACST 189    IQEILPVLEQV.....VQQGRPLIIAEDVEGEALATLVVVKLRGTF
CH60_BACSU 188    IQEILPVLEQV.....VQQKPLIIAEDVEGEALATLVVVKLRGTF
CH60_AGRU 191    LQAMLPVLEAV.....VQTGKPLVIIAEDVEGERLATLVVVKLRGGL
B36917 191       LQAMLPVLEAV.....VQTGKPLVIIAEDVEGERLATLVVVKLRGGL
PAU17072 191     IREMLPVLEAV.....AKAGRPLIIAEDVEGEALATLVVNNMRGIV
CH60_RHILV 191    LQSMPLPVLEAV.....VQTGKPLIIAEDVEGEALATLVVVKLRGGL
CH61_STRCO 187    IADLLPILLEKVI.....QANASKPLIIAEDLEGEALSTLVVVKIRGTF
CH60_COXBU 191    IRELIPLENV.....AKSGRPLIIAEDIEGEALATLVVNNIRGVV
CH62_RHIME 191    LQAMIPILESV.....IQSGKPLIIAEDVEGEALATLVVVKLRASL
PSEGR0ESL1 191    IREMLPVLEAV.....AKAGRPLIIAEDVEGEALATLVVNNMRGIV
CH61_SYN3 189    VQDLVPVLEQV.....ARQKPLIIAEDIEKEALATLVVNRRLRGVL
CH60_CLOTH 189    IQDILPILLEQI.....VQQKKLVIIAEDVEGEALATLVVVKLRGTF
CH60_PSEPU 191    SRAAASATERA.....SAGRPLIIAEDVEGEALATLVVNNMRGIV
CH60_SYN7 190    VQDLVPVLEQV.....ARAGRPLVIIAEDIEKEALATLVVNRRLRGVL
CH60_GALSU 190    VQDILLPVLEQI.....AKTNKPLIIAEDIEKEALATLVVVKLRGIL
CH60_ZYMO 190    LQSILPILESV.....VQSGRPLIIAEDIEGEALATLVVVKLRGGL
JC2564 191       LQSILPILESV.....VQSGRPLIIAEDIEGEALATLVVVKLRGGL
CH60_CHRVI 191    IRDLLPVLEGV.....AKAGKPLIIAEDVEGEALATLVVNTIRGIV
CH60_MYCTU 189    VKDLLPILLEKVI.....IGAGKPLIIAEDVEGEALSTLVVVKIRGTF
CH60_NEIME 191    IRDLLPVLEKV.....AKASRPLIIAENVEGEALATLVVNNIRGIL
CH60_TREPA 189    MKDLLPILLEKI.....AQTRPLIIAEDVEGEALATLVVNSLRGTL
CH60_HELPY 190    MKDILPILLEKT.....MKEGKPLIIAEDIEGEALTTLVVVKLRGVL
CH60_NEIGO 191    IRDLLPVLEQV.....AKASRPLIIAEDVEGEALATLVVNNIRGVL
CH61_CUCMA 222    INAVVKVLELA.....LKKQRPLIIVSEDESEALATLVVVKLRAGI
CH60_MYCPA 189    VKDLLPILLEKV.....IQAGKPLIIAEDVEGEALSTLVVVKIRGTF
MPU15989 230     VKDLLPILLEKV.....IQAGKPLIIAEDVEGEALSTLVVVKIRGTF

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Figure 9(b)

S26582_224	MHAVVKVLEMA.....	LKKQKPLLI	VAEDVESEALGT	LIINKLRAGI
S40247_191	IRDLLPVLEQV.....	AKASRPLLI	IAEDVEGEALAT	LVVNNIRGVL
CH60_CIOAB_189	IQEILPPLEQI.....	VQQKKLLI	IADDDVEGEALAT	LVVNNKLRGTF
CH60_NEIFL_191	IRDLLPVLEQV.....	AKASRPLLI	IAEDVEGEALAT	LVVNNIRGIL
CH60_LEGPN_190	IREMISVLEGV.....	AKSGRPLLI	IAEDVEGEALAT	LVVNNMRGIV
CH62_CUCMA_222	INSVVKVLELA.....	LKRQRPILL	IVSESDALAT	LIINKLRAGI
CHTGROESL1_191	IKDFLPVLEQV.....	AESGRPLLI	IAEDIEGEALAT	LVVNNIRGGF
S40172_64	IKDFLPVLEQV.....	AESGRPLLI	IAEDIEGEALAT	LVVNNIRLRAF
SYOGROEL2_189	IQDLIPTLERV.....	ARSGRPLVI	IAEDVEGEALAT	LVVNNKLRGVL
CH60_CHLPS_191	IKDFLPVLEQV.....	AESGRPLLI	IAEDIEGEALAT	LVVNNIRLRAF
CH62_STRAL_188	VKDLLPPLEKV.....	MQSGKPLLI	IAEDVEGEALST	LVVNNKIRGTF
CH62_MYCLE_189	VKDLLPPLEKV.....	IQAGKSLLI	IAEDVEGEALST	LVVNNKIRGTF
MSGANTM_236	VKDLLPPLEKV.....	IQAGKSLLI	IAEDVEGEALST	LVVNNKIRGTF
CH60_BORBU_190	IKELLPVLEKV.....	LGTNKPLLI	IAEDIEGDALAA	LVVNSVRGAL
S26583_224	MHAVVKVLEMA.....	LKKQRPILL	IAEDVESEALGT	LIINKLRAGI
A49209_190	IKELLPVLEKV.....	LGTNKPLLI	IAEDIEGDALAA	LVVNSVRGAL
MZECPN60B_224	MHAVVKVLEMA.....	LKKQRPILL	IAEDVESEALGT	LIINKLRAGI
CH60_THEP3_189	IQELLPALEQV.....	VQQGRPLLI	IAEDVEGEALAT	LVVNNKLRGTF
CH60_STAEP_188	FQDILPPLEQV.....	VQASRPILI	VADEVEGDALTN	IVLNNMRGTF
CH60_LACIA_189	IQEILPPLEQI.....	LKTNRPILL	IVADDDVEGDAL	PTLVNNKIKGVF
CH61_STRAL_188	IQDILLPLEKVI.....	QAGGSKPLLI	IAEDVEGEALST	LVVNNKIRGTF
CH60_CHLPN_191	IKDFLPVLEQV.....	AESGRPLLI	IAEEIEGEALAT	LVVNNIRLRAF
MZECPN60A_224	MHAVVKVLEMA.....	LKKQKPLLI	VAEDVESEALGT	LIINKLRAGI
HECHSPAB1_190	MKDILPPLEKT.....	MKEGKPLLI	IAEDIEGEALT	TLVVNNKLRGVL
CH60_ARATH_221	INSIVKVLELA.....	LKRQRPILL	IVSESDALAT	LIINKLRAGI
CH60_MAIZE_224	MHAVVKVLEMA.....	LKKQKPLLI	VAEDVESEALGT	LIINKLRAGI
CH60_CHLTR_190	IKDFLPVLEQV.....	AESGRPLLI	IAEDIEGEALAT	LVVNNIRGGF
CH60_STAAU_189	FQDILPPLEQV.....	VQSNRPILI	VADEVEGDALTN	IVLNNMRGTF
CH60_CLOPE_189	IQDILLPLEQI.....	VQAGKLLI	IADDDIEGEAMT	TLVVNNKLRGTF
HS60_YEAST_212	IQDILPALEIS.....	NQSRPPLLI	IAEDVDGEALAA	CILNNKLRGQV
CH60_PYRSA_217	VQELVPIHLELI.....	SKTSRPLLI	IAEDVEKEALAT	LVVNNKLRGIV

Figure 9(c)

CH60_EHRCH_191	IQPILPILENV.....	ARSGRPLLI	IAEDVEGEALSTLVNKLRRGGL
CHTGOE1_191	IKDFLPVLOQV.....	AESGRPLLI	IAEDIVGEALATLVVNRIRGGF
CH60_THETH_188	VRELLPILEQV.....	AOTGKPLLI	IAEDVEGEALATLVVNKLRGTL
TAU294831_189	VRELLPILEQV.....	ROTKGPLLI	IAEDVEGEALATLVVNKLRGTL
CH60_RICTS_190	VQPLVPVLEAV.....	AHTGKPLVLI	ADDDGEALTALILNNLKSGI
SYCCPNC_189	IAELVPVLEAV.....	ARAGRPLLI	IAEDIEGEALATLVVNKARGVL
CPU308211_190	VQDLVPVLEQV.....	ARARKPLFI	IAEDIEKEALATLVVNKLGRNL
CH61_MYCLE_189	LPELLPMLEKV.....	TESGKPLLI	IAEDIEGEALATLVVNSIRKTL
PSU21139_239	ARDLINILEDA.....	IRSGFPVIV	IAEDIEQEALATLVVNLKRGSL
CH60_COWRU_191	IQPLLPILENI.....	ARSGRPLLI	IAEDVEGEALSTLVNKLRRGGL
RUBB_BRANA_245	ARDLVGVLEDA.....	IRGGYPILI	IAEDIEQEALATLVVNLKRGTL
SCCPN60_144	ARDLINVLEEA.....	IRGQYPILI	IAEDIEQEALATLVVNLKRGSL
CH60_EHRI_153	IQPMVPLETTI.....	VRSNRAVVI	IAEDVEGEALTSLVLSKMRGSL
RUBB_ARATH_245	ARDLVGVLEDA.....	IRGGYPILI	IAEDIEQEALATLVVNLKRGTL
ATU49357_235	IKDIIPILEKT.....	TOLRAPLLI	IAEDVTGEALATLVVNLKRGVL
RUB1_BRANA_195	IKDIIPILEKT.....	TOLRAPLLI	IAEDVTGEALATLVVNLKRGVL
CH62_SYNY3_189	IAELVPVLEAV.....	ARAGRPLLI	IAEDIEGEALATLVVNKARGVL
RUBA_RICCO_178	IKDIIPLLEKT.....	TOLRAPLLI	IAEDVTGEALATLVVNKMRGIL
CH60_ODOSI_190	VQDILLPILEQI.....	TKTKRPLLI	IAEDVEKEALATLILNKLRGIV
PSU21105_236	IKDIIPLLEKT.....	TOLRAPLLI	ISEDITGEALATLVVNLKRGIL
CH60_BRANA_224	LNSMVKVLELA.....	LKSORSLLI	IVAADLES DALAVLILNKL RAGI
PMSARG2_105	IADLVPVLETV.....	QKSSSPLVI	IAEEVDGEALATLVVNKNRGVL
RUB2_BRANA_234	IKDIIPILEKT.....	TOLRAPLLI	IAEDVTGEALATLVVNKLRRGVL
CRECPN1A_75	ARDIITILESA.....	IRGNYPLLI	IMAEVEQEALATLVVNKLRRGTL
P60_CRIGR_215	VQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGL
CRECPN1B_224	IRDIIPILEQV.....	TRLNAPLLI	IAEDVSGEALATLVVNKLRRGVL
RUBA_WHEAT_191	IKDIIPLLEQT.....	TOLRCPLFI	VAEDITGEALATLVVNKLRRGII
B47292_189	LPDLLPLEKV.....	AGTGKPLLI	IAEDVEGEALATLVVNNAIRKTL
CELHSP60CP_206	VQDIVPALELA.....	NKLRRPLVI	IAEDVDGEALTTLVNLRLKVGL
P60_HUMAN_215	IQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGL
P60_MOUSE_215	VQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGL

Figure 9(d)

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P60_RAT_215	VQSIVPALEIA.....NAHRKPLVIAEDVDGEALSTLVNRLKVGL
A41931_215	VQSIVPALEIA.....NAHRKPLVIAEDVDGEALSTLVNRLKVGL
MMHSP60A_197	VQSIVPALEIA.....NAHRKPLVIAEDVDGEALSTLVNRLKVGL
CH63_HELVI_218	ASQVVPALALA.....NSQKKPLVIAEDVDGEPLSVLVNKLKIGL
EGHSP60GN_205	IQQILPVLEHI.....AKANRPLVIAEDVDSEALATLIINKINGKL
HS60_SCHPO_222	VQDILPSLELA.....AQRRRPLVIAEDVDGEALAACILNKLRGQL
S61295_198	IHTILPALNHV.....VGTGRPLLIADDDVESEALTTMIFNKLQGKL
TRBMTHSP_198	IHTILPVLNHV.....VRSGRPLLIADDDVESEALTTMIFNKLQGKL
ENHCPN60P_142	SQSVVPLELCL.....LKRKRPLVVISDTIDGDALTTLVLNKLRLGL
CH60_PLAFG_257	IQSILPILEIFAKNKQPLCIADDFSNEVLQTLIINKLKGAIKVLCIVTNSKYISADVGLDLNHNHNMSSFD
CH60_MYCGE_189	IKEILPLLEGS.....VENGNPILLIVAPDFAEEVTTTAVNKLRTGTI
HTOHSP60X_228	VQDIIPALEAS.....TTLRRPLVIAEDIEGEALAVCILNKLRRPT

Figure 9(e)

# INTERNATIONAL SEARCH REPORT

Intern al Application No  
PCT/GB 96/02980

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N15/70 C12N1/21 C07K1/113 C07K14/245  
C07K16/12 A61K38/16 //(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 650 975 A (NIPPON OIL CO LTD) 3 May 1995 cited in the application see claims; examples ---	1-7,11, 12,18, 38-40
X	FEBS LETTERS, vol. 336, no. 2, 27 December 1993, AMSTERDAM NL, pages 363-367, XP002037451 Y. MAKINO ET AL.: "Truncated GroEL monomer has the ability to promote folding of rhodanese without GroES and ATP" cited in the application see page 365, right-hand column, paragraph 2 - page 366, right-hand column, paragraph 1 --- -/--	1-7,11, 12,38-40

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

12 August 1997

Date of mailing of the international search report

1 0. 09. 97

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Fuhr, C



# INTERNATIONAL SEARCH REPORT

Intern: J Application No  
PCT/GB 96/02980

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 35, 1 September 1995, BALTIMORE, MD US, pages 20404-20409, XP002037452 Z.W. WHITE ET AL.: "A Monomeric Variant of GroEL Binds Nucleotides but Is Inactive as a Molecular Chaperone" see page 20407, right-hand column, paragraph 2 - page 20409, left-hand column, last paragraph ---	2
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 11, 18 March 1994, BALTIMORE, MD US, pages 8529-8534, XP002037453 H. TAGUCHI ET AL.: "Monomeric Chaperonin-60 and Its 50-kDa Fragment Possess the Ability to Interact with Non-native Proteins, to Suppress Aggregation, and to Promote Protein Folding" cited in the application see page 8533, left-hand column, paragraph 2 - page 8534, right-hand column, paragraph 1 ---	1-7
T	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 26, 24 December 1996, WASHINGTON US, pages 15024-15029, XP002037454 R. ZAHN ET AL.: "Chaperone activity and structure of monomeric polypeptide binding domains of GroEL" see the whole document -----	1-50

INTERNATIONAL SEARCH REPORT

national application No.

PCT/GB 96/02980

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 47-50  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 47-50  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☒ Claims Nos.: 3-6, 14-22, 24-50  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:  
In view of the extremely large number of compounds falling under claims  
3-6 and claims 14-22, 24-50 referring to them and of the absence of any  
technical support for these compounds in the description, the ISA considers
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

that it is not economically reasonable to draw a search report covering the entire subject matter characterized above.

The search has therefore been limited to the real examples given in the description and closely related ones and includes compounds having the alleged activities.

### Information on patent family members

PCT/GB 96/02980

Form PCT/ISA/210 (patent family annex) (July 1992)